Investigations into Response of Potato to Cadmium with Special Emphasis on Genotypic and Somaclonal Variations

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A thesis submitted in partial fulfilment of the requirements for the Degree of Doctor of Philosophy in Plant Biotechnology

by

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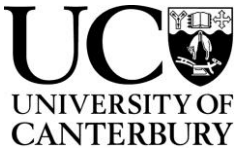

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Tables of Contents

Page

Chapter 2- Variation in Cd Content in Tubers of Selected Potato Genotypes

Chapter 3- Investigations to Generate Potato Cell Lines and Plant Regenerants with Increased Cadmium Resistance

Chapter 5 – Biochemical and Microscopic Studies on the *In Vitro* Selected Cd-Resistant Potato Lines

Chapter 6. Summary, Conclusion and Future Directions

List of Figures

List of Tables

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Abbreviations

%	percent
ºC	degree Celsius
2, 4-D	2, 4-Dichlorophenoxyacetic acid
ABC	ATP-binding cassette
AFLP	amplified fragment length polymorphism
ANOVA	analysis of variance
As	arsenic
Asc	ascorbate
BA	6-benzyladenine
Ca	calcium
CAT	catalase
Cd	cadmium
CFC	cadmium safe cultivar
cm	centimeter
Cu	copper
cv	cultivar
Cys	cysteine
DNA	deoxyribose nucleic acid
DW	dry weight
EDTA	ethylenediaminetetraacetate
EF	enrichment factor
e.g.	example given
FAO	Food and Agriculture Organisation
Fe	iron
Fig	figure
FW	fresh weight
GA3	gibberellic acid
GMO	genetically modified organism
GPX	guaiacol peroxidase
GR	glutathione reductase

GSH	reduced glutathione
GSSG	oxidised glutathione
h	hour
H^+	hydrogen ion
HCl	hydrochloric acid
Hg	mercuric
HNO_3	nitric acid
H_2O	water
H_2O_2	hydrogen peroxide
HSP	heat shock protein
ICP-MS	Inductively Coupled Plasma Mass Spectroscopy
Kg	Kilogram
KI	potassium iodide
KIN	kinetin
KOH	potassium hydroxide
max	maximum
MDA	monodehydroascorbate
mg	milligram
min	minute
mm	millimeter
Mn	manganese
MPC	maximum permissible cadmium
MS	Murashige and Skoog
MT	metallothionine
µg	microgram
µM	micromolar
N	nitrogen
NAA	α-naphthaleneacetic acid
NADPH	nicotinamide adenine dinucleotide
NaCl	sodium chloride
NFHSC	National Food Hygiene Standard of China
nm	nanometer
$O2^{-}$	superoxide anion radical

$OH^{\cdot}$	hydroxyl radical
OSO_4	osmium tetroxide
Pb	lead
PC	phytochelation
PCR	polymerase chain reaction
PGR	plant growth regulator
PIC	picloram
psi	per square inch
PVP	polyvinyl polypyrrolidone
QTL	quantitative trait loci
RAPD	random amplified polymorphic DNA
RFLP	restriction fragment length polymorphism
RGR	relative growth rate
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
SE	standard error
SOD	superoxide dismutase
SSR	single sequence repeats
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TEM	Transmission Electron Microscope/Microscopy
TF	translocation factor
v/v	volume/volume
w/v	weight/volume
WHO	World Health Organisation
Zn	zinc

Publications arising from this thesis

- **Ashrafzadeh S.**, Leung W.M. D., Gaw S., Glover N. Ch. (2015) Differential cadmium resistance of two morphologically distinct types of potato (*Solanum tuberosum*) callus. *Biologia*, 70(5): 581-587.

- **Ashrafzadeh S.**, Leung W.M. D. (2015) *In vitro* breeding of heavy metal-resistant plants: a review, *Horticulture, Environment, and Biotechnology*, 56(2): 131-136.

- **Ashrafzadeh S.**, Leung W.M. D. (2015) Microtuber formation in potato callus. *Scienceasia*, 41(1): 1-4.

Abstract

Tuber crops such as potato (*Solanum tuberosum* L.) can take up high levels of soil cadmium (Cd) which can be accumulated in their tubers. Thus, they can act as vehicles for transporting Cd to human body which can seriously threaten our health due to its high toxicity. In some circumstances, consumption of potato can contribute to more than 50 percent of human dietary Cd intake. In the present research two approaches were used to probe the potential for genetic improvement to contribute towards the goal of "minimisation of Cd level in potato" which is a novel food safety strategy: (1) assess the natural occurrence of variation in Cd accumulating potential among different potato cultivars already in cultivation in New Zealand, and (2) as a proof-of-concept study to generate potato plants with improved Cd resistance from a model experimental potato cultivar Iwa based on plant cell culture-selection (*in vitro* breeding approach).

In the first approach, 10 New Zealand cultivars, namely Red Rascal (RR), Russet Burbank (RB), Fianna (F), Agria (A), Laura (L), Purple Heart (PH), Purple Passion (PP), Yukon Gold (YG), Moonlight (M) and Summer Delight (SD) were chosen randomly among over 30 cultivars grown for seed production in a block situated approximately one kilometre east of Lincoln, Canterbury within the 2011-2012 growing season. The tubers as well as the soil samples immediately surrounding the tubers were harvested and prepared for analytical analyses using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). The results showed that the soil samples were low in Cd level (0.06 mg kg^{-1}), compared with the national soil average (0.35 mg kg^{-1}), while the tubers varied widely in Cd content from 0.04 to 0.34 mg kg^{-1} among different cultivars. Therefore, SD with the lowest mean Cd content (0.05 mg kg^{-1}) and an Enrichment Factor (EF) of just below one showed promise as a potential cadmium safe cultivar (CSC). There might be of concern if L, YG, PP with the

highest mean Cd contents (0.18-0.21 mg kg^{-1}) are grown in soils with higher Cd levels.

Potato tissue culture required for the second approach based on somaclonal variation was established from the model experimental genotype Iwa in the lab. Leaf and internode explants were used as the starting plant materials to initiate two morphologically distinct calli (type-A and -B). Upon morphological assessment and analysis of antioxidative enzymes such as peroxidase, it was revealed that they exhibited differential Cd sensitivity. The more Cd-resistant callus type (type-B) was chosen for *in vitro* selection using 18 different Cd treatments varying in Cd exposure timing and duration. Following shoot and root regeneration from these calli, 18 different new Iwa plant lines were obtained. After at least three months of sub-culturing of all 18 plant lines on Cd-free media, *in vitro* screening of the lines was carried out to identify the most promising plant lines as far as Cd resistance was concerned. After two rounds of *in vitro* growth screening under a low and high Cd levels, two lines including line 9 (L9) and line 11 (L11) were found to exhibit enhanced Cd resistance compared to control Iwa plants. Further studies of L9 and L11 compared to control Iwa plants including biochemical analysis of reactive oxygen species such as hydrogen peroxide, and transmission electron microscopic studies uncovered that L11 was more resistant to Cd than L9 and control plants. L11 plantlets had about 20 and 10 percent less H_2O_2 level than control and L9 plantlets, while antioxidative activities were four and two times higher in L11 compared with control and L9, respectively. Moreover, L11 seemed to exhibit a high rate of Cd compartmentalisation in the vacuoles and Cd binding to the cell walls in the roots, suggesting a potential to exclude or limit Cd translocation to other parts of the plant.

Chapter 1- General Introduction

1.1. Heavy metal pollution

The notable increase in soil pollution with heavy metals (HMs) has become an important public health and food production concern in the past few decades (Sharma and Agrawal, 2005). This issue mainly arises from increased anthropogenic activities such as industrial and agricultural developments added to the levels released from bedrocks (McLaughlin et al., 1999). Some HMs including cadmium (Cd), lead (Pb), arsenic (As), and mercury (Hg) are non-essential for plant growth but very toxic to living organisms even at low concentrations. However, essential HMs such as zinc (Zn), iron (Fe), copper (Cu), and nickel (Ni) can also be harmful at very high levels. Unfortunately, there is no natural remedy for this type of pollution as HMs are persistent in the environment and do not undergo microbial degradation with the passage of time.

Many strategies have been used to remedy soil HM pollution, but none of them could address the problem properly (Wuana and Okieimen, 2011). For instance, application of conventional methods such as soil washing cannot be always feasible since its excavation process is very costly and the risk of spreading of contaminations always exists. Employment of *in situ* techniques such as HM immobilisation by chemical treatments has been found as a practical alternative to render HMs to their non-bioavailable forms without the need for soil excavation. However, detailed knowledge about the relevant soil chemistry is required for their successful application and there is still the concern that the contaminants still remain in soils.

More recently, the use of plants to clean up pollutants from soils (phytoremediation or phytotechnologies) has gained a lot of attention due to its cost effectiveness and aesthetic aspect (Leung, 2013). Conceivably, the various ways in which plants could be used in the management of HM pollution problems may include phytoextraction (to reduce soil HM levels), phytostabilisation (to reduce soil erosion, leaching, and runoff of HMs by *in situ* immobilisation with the help of plant root chemistry), and phytovolatilisation (uptake by roots from soils and then converted by plant cells to volatile forms that can be released into the atmosphere) (Pilon-Smits, 2005). However, many challenges still remain for phytoremediation of soil HM contamination to become of practical relevance in any significant scale globally. For example, there is a limited number of known metal hyperaccumulator plant species and also unprofitability of their products often nullify the practical significance of phytoremediation at present.

To address these disadvantages, the controversial solution of "application of crop hyperaccumulator in phytoremediation" has been proposed by few scientists recently (Hongbo et al., 2011). It has been argued that phytoremediation by non-crop hyperaccumulators is an unrealistic approach due to the preciousness of our finite farmlands which should be only used for the culture of edible plants highly demanded by the world's growing population. These authors also believe that as long as HMs are not accumulated in the edible parts such as grains, there should be no risk to crop yield and safety. To support this, they have suggested symbiosis of crop (legumes) plants with appropriate types of bacteria might help HM accumulation in root nodules (Göhre and Paszkowski, 2006; Wu et al., 2006). However, this is only a hypothesis which has to be shown to be practical. Furthermore, even if these crops can be commercialised after passing a safety

evaluation procedure, achievement of the public acceptance could be another obstacle.

1.2. Sources of soil cadmium (Cd)

Significant quantities of Cd can occur naturally by erosion of ores as almost all of them, particularly, zinc ores contain Cd as a minor component (Babula et al., 2012). However, the increase in soil Cd level is usually a consequence of human activities including mining, disposal of industrial and municipal wastes, manufacturing and disposal of Cd-containing batteries, and application of sewage sludge and fertilisers. In low-industrialised countries such as New Zealand which is agriculturally-intensive, the main source of soil Cd pollution is phosphorus-based fertilisers (Hartke et al., 2013; McDowell et al., 2013).

Phosphate rocks basically contain relatively high levels of Cd, and up to 300 milligram Cd may be present per kilogram phosphate-based fertilisers (Soler and Rovira, 1996). As most of the New Zealand soils are phosphate (P) deficient, agriculture relies on phosphatic fertiliser input. Over the five year period from 2001 to 2005, about 150 tonnes of Cd had been added to New Zealand agricultural soils which is equivalent to approximately 2380 mg Cd added per hectare annually (CadmiumWorkingGroup, 2008; Singh and McLaughlin, 1999).

1.3. Cadmium in soil solutions

Cadmium has high solubility and stable oxidation state ($^{2+}$) in aqueous solutions (Callender, 2003). Cadmium in the solution phase can be as a free hydrated ion which is the most available form for plant uptake or dissolved

species to form complexes with organic or inorganic ligands. The simplest complex is ion pair formed by bonding of a cation (Cd^{2+}) with an anion including chloro complexes which are the most common complex species in soil solutions, particularly, saline soils. The other possible complexes are namely cadmium sulphate ($CdSO_4$) and cadmium carbonate ($CdCO_3$) (Singh and McLaughlin, 1999).

1.4. Cadmium uptake by plants

Uptake of cadmium by plant roots is highly dependent on the soil Cd concentration and also its bioavailability which is affected by soil pH, redox potential, and temperature (Benavides et al., 2005). Hence, rhizosphere can play a key role in HM uptake process as it is the environment in which plants and soils interact. For instance, acidified rhizosphere occurred naturally or by secretion of carboxylates from plant roots, can increase Cd uptake by plants (Benavides et al., 2005). On the other hand, exudation of organic acids including oxalic acid, malic acid, and citric acid which can chelate to Cd, can reduce its solubility and bioavailability and then uptake (Nazar et al., 2012). As a matter of fact, the formed complexes are hardly able to enter into the root cells (Nazar et al., 2012). In addition, concentrations of other trace elements, particularly, bivalent metals such as Ca^{2+}, Mg^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, and Ni^{2+} can affect cadmium uptake as they may compete for being taken up through the same transmembrane transporters (Benavides et al., 2005; Nazar et al., 2012). For example, IRT1 is a well-known multi-specificity transporter from the Zrt/IRT-like protein (ZIP) family. It is mainly expressed in the root cells to facilitate uptake of Cd, Fe, Zn, and Mn (Hall and Williams, 2003).

Once cadmium ions enter the root epidermal cells by extracellular (apoplastic) and/or intracellular (symplastic) pathways, they form carbonate, sulphate, or

phosphate precipitates which are immobilised in the cortex (Lyubenova and Schroeder, 2010). To diffuse into the xylem, the solutes need to drive through the symplasm as the cell wall of endodermal cells is a barrier for them. That would be one of the reasons for retaining of a big portion of Cd ions in the root cells. Cadmium ions which are not accumulated in the root cells can be transported to the other plant parts through the vascular system (Benavides et al., 2005; Lyubenova and Schroeder, 2010).

1.5. Cadmium translocation and partitioning in plants

Cadmium translocation starts with loading in xylem sap which is largely mediated by membrane transporter proteins. Transpiration stream drives the uptake of Cd into the shoot through the xylem and then it is uploaded into apoplasmic spaces of leaf mesophyll cells. This process is facilitated by the tendency for Cd to bind to the sulphydryl-containing ligands such as cysteine in xylem sap. In order for Cd to accumulate in the leaf, Cd has to penetrate into the symplast by crossing the plasma membrane (Dunbar et al., 2003; Singh and McLaughlin, 1999).

Phloem is the only pathway for translocation of Cd from leaf cells to the reproductive organs (Singh and McLaughlin, 1999). Thus, Cd is required to cross the plasma membrane of the companion cells which are adjacent to the sieve tube elements. The low redox potential and alkaline pH of phloem sap make it a very compatible flow to carry Cd complexes. Finally, Cd is unloaded to the terminal reproductive organs including the grains or tubers (Singh and McLaughlin, 1999).

Cd content in these plant parts can vary widely among different plant species. For instance, corn and rice generally accumulate much lower Cd in their seeds than sunflower and flax (Singh and McLaughlin, 1999). Potato tuber, which is

a modified stem, is also found as a strong Cd accumulator structure containing up to 75 percent of total Cd taken up by the plant (Dunbar et al., 2003).

1.6. Cadmium effects on plant growth

Cadmium can negatively affect higher plants. Leaf curling, chlorosis, necrosis, and stunting are some of the morphological effects resulting from Cd exposure. These effects are triggered by a series of changes in plant physiological, biochemical, and molecular profiles imposed by Cd stress (Das et al., 1998). (Toppi and Gabbrielli, 1999).

The inhibitory role of Cd in photosynthesis has a significant negative impact on the physiological functionality of higher plants. It occurs mainly due to damages to the photosynthetic apparatus, lowering chlorophyll and carotenoid contents, and disturbance in actions of photosynthetic enzymes (Toppi and Gabbrielli, 1999). Cadmium can also interact with water balance, alter nutrient uptake, and inhibit stomatal opening and mitochondrial phosphorylation (Nazar et al., 2012; Toppi and Gabbrielli, 1999). Other Cd-induced structural changes include alternation in transcription and inhibition of ribonuclease activities (Toppi and Gabbrielli, 1999).

Imbalances in the oxidation state are the most documented biochemical change in Cd-stressed plant cells. A previous study showed that exposure of *Arabidopsis* seedlings to 50 μM Cd for 7 days, can impose oxidative stress in the leaf cells by either indirect production of oxygen reactive species (ROS) (e.g. through inducing disturbance in the chloroplasts) or inhibition of activities of antioxidative enzymes and non-enzymatic antioxidants (Drążkiewicz et al., 2007). Excessive production of ROS including superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-) can lead to peroxidation of membrane lipids, protein oxidation, damages to nucleic

acids, and even cell death (Benavides et al., 2005; Gill and Tuteja, 2011). Any genetic alternation such as cell division disorder, nucleolus damage, or point mutation, can be inherited to progeny.

1.7. Cadmium resistance in plants

Cadmium resistant plants may evoke different mechanisms to survive in the environment with the presence of elevated bioavailable Cd. Basically, the resistance can be achieved through regulating uptake or/and partitioning of Cd or/and increasing the plant cell tolerance to the Cd-induced oxidative stress. Antioxidative defence is the main response of plant cells to abiotic stresses including heavy metal stress by activation of antioxidative enzymes counteracting reactive oxygen species (ROS) produced by a stress factor (Hall, 2002; Schützendübel and Polle, 2002) thereby minimising the adverse impacts of oxidative stress on the plant cells (Ernst, 2006). The details of these mechanisms have been described below.

1.7.1. Exclusion

Root cell wall is the first barrier against Cd uptake (Fig. 1) as it is in direct contact with Cd in the soil solution. Hence, some Cd ions are kept out of the root cells by binding to root cell wall in its pectic sites, hystidyl groups or extracellular carbohydrate exudates including callose and mucilage (Toppi and Gabbrielli, 1999). Efficiency of this mechanism is highly correlated with the Cd concentration in the soil solution. For instance, no significant difference was observed in the rate of Cd binding to the cell wall between Cd-tolerant and control *Silene cucubalus* plants exposed to low Cd levels (Hall, 2002; Toppi and Gabbrielli, 1999). However, even at high levels, this strategy, by itself,

cannot stand as a comprehensive Cd resistance mechanism in plants, since the number of absorption sites are limited (Furini, 2012).

1.7.2. Immobilisation

Plasma membrane is the first living structure which can prevent HM entrance to the cytosol (Fig. 1). Although it can be the most efficient plant barrier against HM theoretically, its functionality can be adversely affected by high concentrations of toxic metals such as Cd. This damage may be underlain by different causes including changes in lipid composition and fluidity, inhibition of key membrane proteins (e.g. H^+-ATPase), and oxidation or cross-linking of protein thiols. All these can lead to increase in HM leakage into the cytosol (Hall, 2002; Toppi and Gabbrielli, 1999).

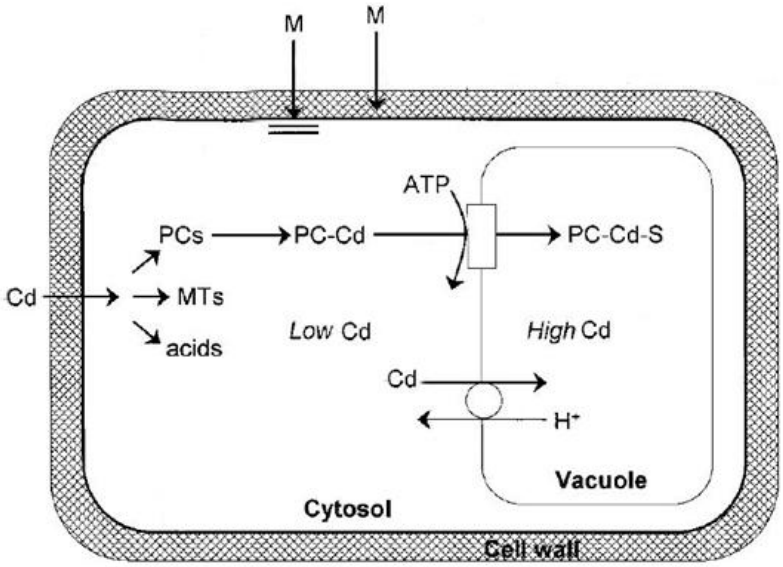


Figure 1. The cellular mechanisms of Cd resistance in higher plants (Hall, 2002) (modified).

1.7.3. Antioxidative defence

It is well understood that abiotic stress factors such as HMs mainly damage plant cells by induction of oxidative stress (Bhaduri and Fulekar, 2012). This is basically arisen from the imbalance in the regeneration and removal of reactive oxygen species (ROS) including hydrogen peroxide, superoxide radicals, singlet oxygen, and hydroxyl radicals (Cho and Seo, 2005). To minimise the oxidative damages, plants may develop the antioxidative defence system involving enzymatic and non-enzymatic antioxidants (Martins et al., 2013).

The main roles of antioxidants are ROS removal and terminating oxidation reactions by being oxidised themselves. The major ROS scavengers are namely superoxide dismutase, catalase, and peroxidises such as ascorbate peroxidise and guaiacol peroxidise. The balance among them is the key point for the plant cells to determine the steady level of ROS. This can lead to a modulation of the oxidative status of the cells and keep it under control (Mittler, 2002).

1.7.3.1. Superoxide dismutase (SOD)

Superoxide dismutase (SOD) (EC 1.15.1.1) is known as the first line of antioxidative defence against ROS. It is a metallo-enzyme catalyzing the dismulation of superoxide free radicals to hydrogen peroxide (H_2O_2) according to the following reaction.

$$2O_2^{\cdot -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

As O_2^- is produced by electron transport chain, SOD is present in many compartments. Based on its metal ion co-factors and location, SOD has been categorised in three types including FeSOD (found in chloroplast), MnSOD (found in mitochondrion and peroxisome), and Cu/ZnSOD (found in chloroplast and cell wall) (Alscher et al., 2002; Bhaduri and Fulekar, 2012). Regarding to the above reaction, an increase in SOD activity can result in an increase in production of hydrogen peroxide. Therefore, activities of SOD and enzymes catalysing H_2O_2 to water (peroxidases and catalase) should be under close control. Hydrogen peroxide level itself can control SOD activity by activating or inactivating the enzyme. However, sensibilities of different SOD isoforms to hydrogen peroxide have been found to be different (Mishra et al., 2006).

Previous studies revealed that Cd effects on SOD isoforms can be species-dependent (Drążkiewicz et al., 2007; Gorinova et al., 2007). For example, Drążkiewicz et al. (2007) measured the activities of different types of SOD in leaves of *Arabidopsis thaliana* exposed to Cd for a week. The results showed that FeSOD and MnSOD activities were increased compared to those in the control, but Cu/ZnSOD activity was decreased. Conversely, Cd stress did not affect FeSOD and MnSOD activities in tobacco leaves whereas it increased Cu/ZnSOD activity (Gorinova et al., 2007).

Cd-induced changes in SOD activity may also vary among different genotypes. For instance, Gonçalves et al. (2009) compared SOD activities of two potato genotypes (Asterix and Mecaca) exposed to different cadmium levels (50, 100, 150, and 200 mg kg^{-1}) for 7 days. The results showed that although Cd inhibited SOD activity in the roots of Asterix at all levels, but increased it in Mecaca roots at the two highest levels (150 and 200 mg kg^{-1}). Similarly, SOD activity in shoot cells of Mecaca decreased, whereas it showed an increase in Asterix at all the Cd levels.

1.7.3.2. Catalase (CAT)

Catalase (CAT) (EC 1.11.1.6) is a heme-containing oxidoreductase found in most plant and animal cells. It acts after the SOD reaction and is responsible for conversion of H_2O_2 to harmless water and oxygen. This enzyme is mainly localised in peroxisomes and activated at high H_2O_2 concentrations. Catalase can play a key role in scavenging H_2O_2 produced during photo-respiration and β-oxidation of fatty acids. However, despite its very high efficiency, it has a low affinity for H_2O_2. This can be due to its requirement to access to two molecules of hydrogen peroxide simultaneously (Bhaduri and Fulekar, 2012; Ridge, 1997).

In fact, hydrogen peroxide is a signal molecule in abiotic stresses. It can activate enzymes involved in antioxidative defence system such as CAT (Martins et al., 2013). Bočová et al. (2012) found that short-term treatment (30 minutes) of barley roots with a moderate level of Cd (30 μM) can increase CAT activity resulting in restoration of root growth. But a severe Cd stress (60 μM) inhibited CAT activity leading to serious damages to root cells (Bočová et al., 2012). Cho and Seo (2005) also reported a decrease in CAT activity caused by H_2O_2 increase in *Arabidopsis* seedlings exposed to 300 and 500 μM Cd for three weeks.

It seems that CAT activity can be genotype-dependent as well (Cho and Seo, 2005). For example, decrease in CAT activity was only seen in roots of Asterix cultivar exposed to 100 and 150 μM Cd whereas this change was reported in both roots and shoots of Mecaca cultivar in response to a wider range of Cd levels (Gonçalves et al., 2009).

1.7.3.3. Peroxidases

Peroxidases (POD) (EC 1.11.1.) is a group of hemeproteins found in plant and animal cells as well as microorganisms. As their levels are generally modified under HM stress, they are known as metal stress-related biomarkers (Jouili et al., 2011). Although like CAT they act as hydrogen peroxide scavengers, they are not polymeric and also have a wider substrate range including mono- and dihydroxy alcohols and phenols, dihydroquinones, amines, and many other hydrogen donors (Ridge, 1997). Thereby, their names are mainly based on substrates which they use. The two main classes of peroxidases found in higher plants are ascorbate peroxidase (APX; EC 1.11.1.11) and guaiacol peroxidase (GPX; EC 1.11.1.7) using ascorbate and guaiacol as the reductants, respectively (Jouili et al., 2011; Martins et al., 2013).

Ascorbic peroxidase is primarily localised in the cytosol and chloroplast of the plant cells (Ridge, 1997). Although it has the same biochemical action as CAT (scavenging H_2O_2), but its higher affinity and different enzymic mechanism distinguish it from CAT. Thus it was thought that APX would mainly be responsible for ROS modulation in signalling process rather than removal of excess ROS formed during stress (Cho and Seo, 2005). However, other studies in higher plants including *Arabidipsis*, tobacco, soybean and maize showed that APX has no clear relationship to Cd stress as its responses to different Cd concentrations were contradictory (Martins et al., 2013).

Guaiacol peroxidase (GPX) is the most important class of peroxidase enzymes in plants. It is mainly located in the vacuole and apoplast but it has been also found in the other cellular compartments including mitochondrion, nucleus, plasma membrane, and cell wall (Martins et al., 2013). Although guaiacol is a

common substrate used in the assay for GPX activity, GPX can also catalyse the oxidation of other phenolic compounds (Martins et al., 2013).

In plants under metal stress, GPX acts as a multifunctional enzyme playing roles in activation of some physiological and biochemical processes as well as H_2O_2 degradation (Jouili et al., 2011). Furthermore, increase in antioxidant capacity of plant cells is the other function of GPX. Cadmium is one of the HMs known as a regulating factor for guaiacol peroxidase genes (Jouili et al., 2011). Therefore, GPX is of high importance to any study in relation to metal stress in plants.

1.7.3.4. Glutathione Reductase

Glutathione reductase (GR) (EC 1.8.1.7) is a flavoprotein catalysing the reduction of oxidised glutathione (GSSG) to its reduced form: glutathione (GSH). Glutathione is highly essential for controlling redox status of cells, phytochelatin (PC) synthesis, and many other biological processes. This reaction mainly takes place in the cytosol and mitochondrial matrix at the expense of one NAD(P)H. Glutathione reductase is also used as the substrate for glutathione peroxidase to reduce H_2O_2 and organic peroxides in plants under stress (Ridge, 1997).

Cadmium decreased GR activity in wild and tolerant lines of *Arabidopsis* seedlings, but the level of GR activity was much higher in the tolerant line than wild type. This might be related to the contribution of GR in Cd resistance mechanism (Cho and Seo, 2005). The reason of the decrease in the GR activity can be the active synthesis of PC leading to GSH depletion or direct inactivation of thiol-containing enzymes including GR (Cho and Seo, 2005; Martins et al., 2013).

1.7.4. Organic acids and amino acids

Organic acids including citric and malic acids and amino acids such as histidine are also involved in metal detoxification. They can chelate with HMs which have affinity to N, O, or S (Clemens, 2001; Hall, 2002). For instance, citric acid has been known as the main Cd ligand at low Cd levels (Wanger, 1993). X-ray absorption spectroscopy in *Brassica juncea* revealed that organic acids are found more often in the xylem rather than the root cells where PCs are in charge to act in a similar chelating role (Salt et al., 1995).

1.7.5. Stress proteins

Stress proteins are a group of polypeptides in different organisms in response to a variety of abiotic stress factors such as high temperature, drought, or heavy metals. They are generally classified based on their molecular weights. In plants, stress proteins with small sizes are more efficient than big proteins. For instance, ubiquitin is a highly conserved active protein with only 76 amino acids (Hall, 2002; Toppi and Gabbrielli, 1999).

Stress proteins generally have two functionalities. One of them is acting as molecular chaperons in protein folding and assembly process. For example, HSP70 showed a strong affinity to refold the misfolded proteins to their native conformations (Toppi and Gabbrielli, 1999). Moreover, they are able to protect and repair proteins exposed to different stresses. However, the exact functionality and mechanism of many stress proteins are still unknown (Hall, 2002; Toppi and Gabbrielli, 1999).

Expression of these proteins can be regulated by specific transcripts found in Cd-stressed cells (Czarnecka et al., 1984; Edelman et al., 1988; Schoeffl and Key, 1982). For instance, Cd-exposed cells of soybean were able to regulate

the synthesis of stress proteins by inhibition of splicing in *Gmhsp26-A* pre-mRNA (Czarnecka et al., 1988). However, the role of the introns in this regulation is not clear yet (Toppi and Gabbrielli, 1999).

1.7.6. Compartmentalisation

Sequestration of HMs in specialised organelles such as vacuoles is a key tolerance mechanism minimising the adverse impacts of HM stress in the cytosol (Fig. 1). Thereby, many cytoplasmic functional activities as well as vital organelles including chloroplast and nucleolus, can be kept away from HM stress (Toppi and Gabbrielli, 1999).

Application of electron microscopy has helped to understand HM intra-cellular localisation more precisely. For instance, transmission electron microscopic (TEM) analyses on oilseed rape (*Brassica nupus)* grown in Cd-contaminated soils showed that the vacuole is the main Cd store, whereas only little Cd was seen in the chloroplasts (Carrier et al., 2003). Another metal localisation study in barley leaves revealed that the vacuoles contained the main proportion of total Cd, Zn, and Mo in the cells (Brune et al., 1994). Transport of Cd from cytosol into the vacuole is mediated by two types of transporters located within the tonoplast (Fig. 1) (Hall, 2002).

One of the metal transporters is a Cd/H^+ antiporter which is able to mediate transfer of free Cd ions from the cytosol into the vacuole (Toppi and Gabbrielli, 1999). ATP-binding cassette (ABC) proteins are the other transporter that allows Cd ions bound to a chelating peptide called phytochelatin (PC) to enter the tonoplast (Hall, 2002). Afterwards, the Cd-PCs can be stabilised in the vacuole by acquiring acid-labile sulphur (S^{2-}) and then forming high molecular weight (HMW) complexes. It can also increase their affinities towards free Cd ions (Hall, 2002; Toppi and Gabbrielli, 1999).

1.7.6.1. Phytochelatins

Phytochelatins (PCs) are a family of cysteine-rich peptides with the general structure $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n=2-11) which can form complexes with heavy metals (Fig. 1). They can be enzymatically synthesised from glutathione in cytosol upon exposure to HMs, particularly Cd. In fact, phytochelatin synthase is an enzyme activated in plants in response to Cd exposure (Hall, 2002; Toppi and Gabbrielli, 1999).

Phytochelatins are able to form various complexes with Cd based on chelating affinities of the Cys thiol groups. Therefore, they are able to prevent free Cd ions to circulate inside the cytosol. The importance of PCs in Cd detoxification was revealed in an experiment using the *cad1* mutant of *Arabidopsis thaliana* which was deficient in phytochelatin synthase and also Cd-sensitive (Hall, 2002; Toppi and Gabbrielli, 1999). In addition, short-term exposure of potato tubers to Cd led to increases in the *stPCS1* transcript level, phytochelatin synthase activity, and contents of PCs and cysteine (Stroiński et al., 2010).

1.7.6.2. Metallothioneins

Metallothioneins (MTs) are the other family of Cys-rich metal-binding peptides. Cysteine residues of plant metallothioneines are present as Cys-x-Cys, Cys-x-x-Cys or Cys-Cys cluster. Occurrence of MT genes has been documented in a wide range of higher plants (Hall, 2002; Toppi and Gabbrielli, 1999). Zhou and Goldsbrough (1994) reported the increase of *MT2* translation in *Arabidopsis* seedlings exposed to Cu, Cd, and Zn. Although the MT role in Cd detoxification has not been found as important as the PC role, it seems that further investigation is required (Hamer, 1986; Toppi and Gabbrielli, 1999).

1.8. Cadmium effects on human health

Cadmium can pose severe impacts on human health (Satarug et al., 2010). In human body, blood transfers the absorbed Cd in gastrointestinal system to different organs including liver, kidney, testis, spleen, heart, thymus, salivary glands, epididymis, and prostate which can be impaired at high Cd levels (Joseph, 2009). Some of them including liver and kidney can be affected more than others due to their higher capacities in Cd storage. It can be due to high concentrations of metallothionein (MT) in these organs. Approximately 50 percent of the Cd in the body is found in the liver and kidney. It is a serious health concern as Cd half-life in these organs was estimated 15-20 years (Joseph, 2009). Chronic exposure to high concentrations of cadmium can cause *itai-itai* which is a disease impairing tubular and glomerular functions mainly in women and also increase the risk of osteoporosis and bone fractures (Pan et al., 2010; Satarug et al., 2010). Moreover, Cd has been classified as a carcinogen regarding its roles in inhibition of DNA damage repair and induction of apoptosis, oxidative stress, and aberrant gene expression (Joseph, 2009).

1.9. Importance of cadmium resistant potatoes

Potato (*Solanum tuberosum* L.) is a staple crop species of the Solanaceae (nightshades) family. Worldwide, it is the fourth most cultivated crop after wheat, rice and maize with the production of around 323 million tonnes (Lachman et al., 2009). In New Zealand, just over half a million tonnes are produced annually and the value of the potato industry has been estimated to be worth around $500 million.

The potato tuber (the edible part) is actually a modified stolon, containing high levels of starch, which is the most common carbohydrate in human diets. In addition, it can be a rich source of many essential elements including Fe and Zn giving it a high nutritional value. However, its relatively high ability in accumulation of toxic metals such as Cd is a food safety concern. Under some circumstances, potato can comprise more than 50 percent of human dietary Cd intake (Dunbar et al., 2003; Reid et al., 2003). Therefore, it can be a competent vehicle for driving Cd into our bodies.

Regarding the tolerable daily Cd intake of 1 µg per kg body weight recommended by the World Health Organisation (WHO), particular caution is needed with respect to the consumption of this crop (World Health Organisation, 1992).

1.10. Understanding mechanisms of Cd resistance

Plant lines regenerated after *in vitro* selection using Cd supplemented in the growth medium can be further analysed based on *in vitro* screening. This will help to identify the most promising resistant plant lines through their distinct growth performance in the presence of Cd stress compared to the control (or mother plant line used to initiate plant tissue culture for *in vitro* selection). In fact, this method is a relatively quick, easy and clean laboratory-based technique to assess components/compounds under stress (such as Cd) contained in cultivation media (Golan-Goldhirsh et al., 2004). For example, a large-scale *in vitro* screening of 25 commercial flax/linseed varieties was comprised of culturing them in media containing a sub-lethal Cd concentration (170 µM) for three weeks, followed by assessment of number of organogenic points such as buds and shoots as well as dry weights (Smykalova et al., 2010).

However, to understand HM resistance mechanism(s) in the lab , the plants can be studied at different levels including biochemical and physiological. The parameters studied most frequently in the previous biochemical studies were ROS production, activities of antioxidative enzymes, and contents of chlorophyll and stress-related amino acids such as proline (Baisakhi et al., 2003; Samantaray et al., 2001; Shekhawat et al., 2010; Wang et al., 2013; Xu et al., 2009). The results showed that comparison of these parameters between tolerant and non-tolerant plants in different organs can reveal their biochemical differences.

Localisation of HMs in plant organelles and structures can be visualised using powerful microscopic techniques including scanning electron microscopy (SEM) and transmission electron microscopy (TEM) (Prasad and Hagemeyer, 1999). The knowledge about sub-cellular distribution of HMs can greatly help us to understand the physiological aspects of HM tolerance mechanisms involved. For example, Ni et al. (2005) studied localisation of copper (Cu) in a tolerant (*Elsholtzia splendens* Naki*)* and non-tolerant (*Astragalus sinicus* L.) plant species by using TEM. The results clearly pointed to the key roles of the vacuoles and cell walls in Cu tolerance in *E. splendens*.

1.11. Overview of this thesis research

In the recent decades, scientists have attempted to address the food safety concern of HMs which is the other side of phyto-extraction. Hence, instead of soil remediation, they have proposed the culture of low HM accumulating crops to minimise human HM intake through food crops. Clearly, the basic pre-requisite for this novel approach is identification or development of metal-resistant crop plants which can take up low levels of HMs from soils or/and translocate a low portion of HMs taken up by roots to the above-ground plant parts. These strategies can keep photosynthetic organs away from HM stress resulting in sustainability of plant growth (Nocito et al., 2011). Plants which can evoke either of these mechanisms for limiting HM concentrations in the edible plant parts are of high value in terms of food safety.

A literature survey has found evidence for natural variation in HM uptake and partitioning among different plant cultivars as well as species (Grant et al., 2008). This has been further explained in Chapter 2. Based on this, a research goal was to identify crop cultivars with low HM uptake and/or accumulation ability. Screening crop cultivars for HM content is possible using mass spectrometry but this approach can be costly. Inductively coupled plasma mass spectrometry (*ICP-MS*) is one of the most precise trace element analytical equipment which is able to separate and quantify trace elements even at extremely low concentrations (Klotz et al., 2013).

Application of this source of variation in HM accumulation in plants has given rise to novel valuable concepts. Cadmium safe cultivar (CSC) is one of them which has been proposed recently (Liu *et al*., 2010; Zhang *et al*., 2014). The edible parts of these cultivars contain low enough Cd for safe consumption (Yu et al., 2006). On the other hand, identification of crop cultivars with high Cd accumulation can be beneficial as it can guide agricultural industry to avoid

culturing them for consumption purpose, particularly, in countries with Cd-polluted soils. Alternatively, they can be used as hyperaccumulators to clean up Cd contamination of soils (phytoremediation). In general, although this natural variation is not always broad enough to find the plants of interest, it can be extended with the help of traditional plant breeding or biotechnological methods which are able to induce artificial variation.

Biotechnology using plant cell and tissue culture is a powerful tool which can induce variation *via* somaclonal variation phenomenon (Lestari and Endang Gati, 2006). Therefore, the variant plants obtained using plant tissue culture methods are not regarded as GMOs (genetically modified organisms *via* gene manipulations or gene transformation popularly known as genetic engineering) as no *in vitro*-manipulated or recombinant DNA was transferred to generate the variants. Afterwards, variants of interest can be easily selected through this approach under highly controlled conditions. Unlike traditional plant breeding, there is a minimal requirement of space and time for this approach.
Plant tissue culture has been employed to develop resistant plants to different abiotic stresses such as HM stress. This technique is an alternative option for breeders in countries such as New Zealand in which recombinant gene manipulation is under very restricted regulatory criteria. This approach basically starts with initiation of callus cells which are undifferentiated heterogeneous cells capable to achieve new traits following exposure to a stress factor as a selecting agent during callus proliferation. This can increase the chance of somaclonal variation which may lead to development of HM-resistant callus cells. The factors such as ploidy of source material, origin of tissue, and also treatment level and time can affect somaclonal variation (Karp, 1990). Regeneration of new plants from callus cells are possible based on the plant totipotency concept proposed by Haberlandt in 1902. The presence of a selected stress factor (HMs) in plant regeneration media can result in selection

of desirable somaclonal variants. This procedure takes much less efforts and costs than conventional breeding methods. Moreover, as described above, the plants developed through this method have more public acceptance than genetically engineered plants based on recombinant gene transfer. However, in spite of its numerous advantages, it has been barely exploited to generate HM-resistant plants. This has been explained further and the experiments carried out to obtain and study cadmium-resistant potato plants from plant tissue culture were described and discussed in Chapters 3-6.

In summary, as potatoes are highly important in our diets, particularly in Western cuisines, minimisation of potato Cd level seems a promising approach or goal from the food safety perspective. To make progress towards this goal, the following two objectives were targeted, which are based on natural and artificial (somaclonal) variation sources, respectively.

I. Identification of potato cultivars grown in New Zealand that exhibit low Cd accumulation (Chapter 2).
II. *In vitro* selection and screening of Cd resistant potato lines, some of which might have enhanced Cd exclusion capabilities (Chapters 3 & 4).

For a better understanding of the mechanisms of Cd resistance, the new plant lines derived from potato tissue culture were subjected to further biochemical and transmission electron microscopic studies (Chapter 5). In the concluding chapter (Chapter 6), the main findings and experiments worthy of further research were discussed.

Chapter 2- Variation in Cd Content in Tubers of Selected Potato Genotypes

2.1. Introduction

Previous studies revealed that Cd resistance can be cultivar-specific in higher plants (Tables1&2&3). The variation in Cd uptake and accumulation is well-documented among different cultivars of two staple crops: rice (Cheng et al., 2006; He et al., 2006; Hongjiang et al., 2014; Liu et al., 2005; Yu et al., 2006; Zeng et al., 2008; Zhan et al., 2013) and wheat (Gao et al., 2011; Gray et al., 2001; Greger and Loefstedt, 2004; Stolt et al., 2006). Not enough work has been done on the other staple crops including potato, which can comprise more than 50 percent of human dietary Cd intake, however.

Harris and colleagues (1981) found no correlation between the cultivar and Cd accumulation in potato tubers grown in a Cd-contaminated field, whereas McLaughlin et al. (1997) reported a considerable difference in tuber Cd concentrations of 14 most common Australian cultivars. Likewise, Dunbar *et al*. (2003) found different Cd contents in tubers of two Australian varieties: Wilwash and Kennebec. A similar finding was obtained for sixteen potato cultivars grown in Turkey (Ozturk et al., 2011).

It is known that only a small fraction of Cd can be directly absorbed across the periderm to potato tubers and that the main proportion (~85%) of Cd is transferred through the basal roots to shoots *via* the xylem and then back down by phloem to tubers (Reid *et al.*, 2003). According to a previous study, no functional xylem was found between the basal roots and tubers for direct transfer of Cd and other trace elements (Dunbar *et al*., 2003). As the tuber is the final sink for Cd, tuber Cd content will be influenced by physiological aspects of Cd handling, including uptake from soils, translocation and

accumulation into different plant organs (Dunbar *et al*., 2003; Reid *et al*., 2003).

In potato, cultivar-dependent difference in tuber Cd content was suggested to be due to variation in Cd distribution among potato organs rather than a difference in root uptake of Cd (Dunbar *et al*., 2003). It has been shown that the proportions of Cd partitioned in the roots, shoots and tubers of two Australian cultivars (Kennebec and Wilwash) were different while the total Cd uptake into the plants was constant (Dunbar *et al*., 2003). Seventy five percent of the total amount of Cd taken up by Kennebec plants was partitioned in the tubers whereas Wilwash‘s tubers only contained 43 percent. On the other hand, root Cd burden in Kennebec was one third of that in Wilwash. It is possible that the portion of the Cd translocated in the phloem and that was not retained or accumulated in roots and leaves could be transferred into tubers for accumulation. Chen et al. (2014) reported that the potato leaf can accumulate the biggest portion of the uptaked Cd. This will be dependent on the relative capacities of cellular and biochemical mechanisms in the different plant parts responsible for Cd sequestration, and will influence processes such as physical compartmentalisation of Cd (e.g. into vacuoles) and/or complexation of Cd with chemical compounds (Dunbar *et al*., 2003).

Cd uptake is not only important in terms of representing a potentially toxic level, but it may also influence the uptake of other elements, and may thus contribute indirectly to toxicity or, in the case of nutrients, deficiency of these elements. Interaction between the uptake of Cd and other trace elements has been noted previously in plants. Cataldo and Garland (1983) found an antagonistic correlation in accumulation of Cd and Fe, Zn, Cu, Mn in soybean suggesting a common transport site or process might be involved in the uptake of these trace elements. Chen *et al*. (2007) compared the bioaccumulation patterns of Cd and Fe, Zn, Cu, Mn in barley cultivars

concluding that only Mn uptake was correlated significantly with that of Cd. Zhang et al. (2002) found that correlation between uptake and translocation of Cd and some other trace elements was cultivar-dependent in wheat. A synergistic correlation was observed between Cd and Zn uptake in tomato (Smith and Brennan, 1983). Likewise, in potato tubers of two Australian cultivars, Ca, Mg, Mn, Fe, Cu, and Zn contents showed positive relationships with partitioned Cd level (Dunbar *et al*., 2003).

It is well-documented that Cd uptake and accumulation is governed at the molecular level (Benavides et al., 2005; Hall, 2002). A QTL study on 39 chromosomes of brown rice (*Oryza sativa*) discovered chromosomal regions controlling Cd accumulation. They were located on chromosomes 3, 6, and 8 (Ishikawa et al., 2005). Likewise, in wheat and *Arabidopsis*, Cd uptake was found linked to loci on chromosome 5 (Howden and Cobbett, 1992). Furthermore, application of four molecular markers in oat (*Avena sativa*) found the similar major gene controlling grain Cd accumulation (Tanhuanpää et al., 2007). Clarke et al. (1997) also reported a single dominant gene controlling the highly heritable trait of low grain Cd accumulation in durum wheat (*Triticum durum*). Low Cd accumulation in a soybean cultivar (Westag 97) was also found correlated with expression of three genes (Wang et al., 2014b).

Proteins expressed from these regulatory genes can play key roles in controlling metal translocation among different plant organs (Sugiyama et al., 2007). For instance, Ahsan et al. (2012) found highly active proteins and amino acids associated with Cd chelating pathway in root cells of a soybean cultivar (Enrei) translocating a low level of Cd to above-ground organs (Table 2). These genes may also be correlated with the physiology of root cell walls affecting Cd uptake very efficiently (Ovečka and Takáč, 2014).

Besides genotype, different environmental factors including different growing seasons and soil properties such as soil texture, pH, nitrogen (N) level, and Cd and other microelement contents can also affect plant Cd uptake. However, among these factors, soil acidification and high N levels have been found to be more influential on the increase in Cd bioavailability and consequently Cd uptake, particularly in sandy soils (van Gestel, 2008). This condition mainly occurs due to excessive application of N-containing fertilisers (Eriksson, 1990; Zhu et al., 2010). However, the impact of genotype was found to be more significant and consistent by the previous studies summarised in Tables 1, 2 and 3. This can be explained by a study on the model plant (*Arabidopsis thaliana*) showing the substantial impacts of plant traits such as growth ability in Cd uptake and distribution (Dauthieu et al., 2009). The main reason for this attribution may be the dependence of Cd movement to the transpiration process which is highly correlated with development of plant roots and leaves. Therefore, individual characteristics of different genotypes can play roles in Cd uptake and accumulation processes.

Genotype impact on Cd accumulation was identified to be much more effective than soil factors and growing seasons in four spring wheat cultivars grown in three sites in Sweden (Stolt et al., 2006) (Table 1). The observed variation pattern was consistent regardless of differences in seasonal averages of soil Cd levels (0.04 – 0.13 mg kg^{-1}) and pH (5.8 – 6.6) among the different sites. Stolt et al. (2006) suggested that the roots have a more significant role in regulating grain Cd accumulation than shoots as both the shoots and grains of the high accumulating cultivar (Thasos) contained relatively high Cd levels. This also means that shoot Cd content can be an indicator for grain Cd level, so that early selection of high accumulating cultivars may be possible. This can greatly facilitate the selection process and reduce the procedure time. A

low Cd accumulating cultivar (Grandur) was introduced in this study (Table 1).

Table 1. Studies on grain Cd accumulation in Poaceae family.

Species	Origin of cultivars	Number of cultivars	Variation in Cd accumulation (*=significant, n.d.=not determined)		Low-Cd accumulating cultivars **(Bold=CSC)**	High-Cd accumulating cultivars	Reference
Triticum aestivum (wheat)	Sweden	4	*		Grandur	Thasos	(Stolt et al., 2006)
	Sweden	7	*	n.d.	Tjalve	Vinjet	(Greger and Loefstedt, 2004)
	Sweden	4	*	*		Holme	(Andersson and Pettersson, 1981)
	Sweden	4	*	*	Reeves, Kulin	Egret, Oxley	(Kjellstrom et al., 1975)
	Australia	35	*		Monad	Consort	(Oliver et al., 1995)
	New Zealand	43	*	*	AC Cora	Guard, AC Domain	(Gray et al., 2001)
	Canada - USA	13	*				(Gao et al., 2011)
Orizq sativa (rice)	China, Japan, America, Southeast Asia	52	*	n.d	Ming hui 63	Bing 814	(Liu et al., 2005)
	Japan	49	*		Milyang 23, Peh-Kuh-Tsao-Tu	Nipponbare, Koshihikari	(Arao and Ae, 2003)
	Japan	35	*		Mitsuyou 23, Ir8	LAC23, Hu Lo Tao	(Arao and Ae, 2001)
	China	138	*	*	Mingzhu 1	Chunjiang 026, Chunjiang 11, Hu 97 98	(Zeng et al., 2008)
	China	38	*	n.d.	Nanjing 41	Nanjing 40	(He et al., 2006)
	China	9	*	*	Xiushui 110	Chunjiang 101	(Cheng et al., 2006)
	China	39	*	*			(Yu et al., 2006)
	China	43	*	n.d.	**Shendao5, Tianfu1 Fuhe90,Yanfeng47**		(Zhan et al., 2013)
	China	146	*	n.d.	**Lu5278-1332, Lu17-T21712, Lu17-12R60, IRBN95-90, D26B**		(Hongjiang et al., 2014)
Triticum durum (durum wheat)	Sweden	4	*	n.d.		Helidur	(Greger and Loefstedt, 2004)
	Canada	4	*	*		Kyle	(Cieslinski et al., 1996)
	Canada	2	*	*			(Gao et al., 2011)
	USA	30		n.d.	Ward	Medora	(Li et al., 1997)
Zea mays (corn)	China	3	*	*		Yunshi 5	(Guo et al., 2011)
Hordeum vulgare (barley)	China and elsewhere	600	*	n.d.	E-barly 6, Zhenong 8	Beitalys, Shang 98-128	(Chen et al., 2007)
	China	8					(Wu and Zhang, 2002)
	Sweden	5	*				(Kjellstrom et al., 1975)
Lolium prenne (ryegrass)	New Zealand	10	*	*		Tabu	(Gray and McLaren, 2005)

Table 2. Studies on legume Cd accumulation in Fabaceae family.

Species	Origin of cultivars	Number of cultivars	Variation in Cd accumulation (*=significant, n.d.=not determined)		Low-Cd accumulating cultivars	High-Cd accumulating cultivars	Reference
Arachis hypogaea (peanut)	Australia	2	*		NC7	Streeton	(McLaughlin et al., 2000)
	Australia	11	*	*	RMP 91	A166 L17	(Bell et al., 1997)
Glycine max (soybean)	Japan	4	*		Suzuyutaka	Enrei	(Sugiyama et al., 2007)
	Japan	17	*		Harosoy	En-b0-1-2	(Arao et al., 2003)
	Japan	18	*		Harosoy	En-b0-1-2	(Arao and Ae, 2001)
	Japan	150	*		Harosoy		(Sugiyama et al., 2011)
Vigna ynguicular (asparagus bean)	China	24	*	*		Chuangshanjiang 802	(Zhu et al., 2007)
Pisum sativum (pea)	UK	5	*	*	Douce P	Pilot	(Alexander et al., 2006)
Phaseolus vulgaris (french bean)	UK	5					(Alexander et al., 2006)

Table 3. Studies on the Cd accumulation in the edible parts of other family species.

Species	Origin of cultivars	Number of cultivars/lines	Variation in Cd accumulation (*=significant, n.d.=not determined)		Low-Cd accumulating cultivars **(Bold=CSC)**	High-Cd accumulating cultivars	Reference
Lactuca sativa (lettuce)	Wales	6	*		Webb's Wonderful	All Year Round	(Crews and Davies, 1985)
	UK	5					(Alexander et al., 2006)
	China	28	*		**SJLV, SJGC, SJDT YLGC, N518, KR17**		(Zhang et al., 2013)
Linum usitatissimum (linseed)	Australia and elsewhere	17	*	n.d.	Mikael	Argyle	(Hocking and McLaughlin, 2000)
Allium cepa (onion)	UK	5					(Alexander et al., 2006)
Allium fistulosum (Welsh onion)	China	25	*		**Ribentiegancongwang, Wuyeqi**		(Li et al., 2012)
Brassica rapa (Chinese cabbage)	China-Japan	50	*	*	**Hanzhouyoudonger, Aijiaiheiye 333, Zaoshenghuajing**		(Chen et al., 2012)
	China	40	*		**New Beijing 3, Fengyuanxin 3**	Suancaiwang	(Liu et al., 2010)
	China	31	*		**49 Caixin, Chihua NO.4 Youlu 80, Xianggang 49 Lubao 70, 49NO.19**		(Qiu et al., 2011)
Raphanus sativus (radish)	China	12	*	n.d.	Chaojimeinong	Changbai 20 day	(Zheng et al., 2008)
Spinacia oleracea (spinach)	UK	5					(Alexander et al., 2006)
Daucus carota (carrot)	UK	5	*	*	Nairobi	Amsterdam	(Alexander et al., 2006)
	China	10	*	n.d	.Japan 5-inch	Kaqi	(Zheng et al., 2008)
Solanum tuberosum (potato)	Australia	14	*	*	Lemhi Russet	Toolangi Delight	(McLaughlin et al., 1994)
	Australia	2	*	n.d.	Wilwash	Kennebec	(Dunbar et al., 2003)
	Turkey	16	*	n.d.	Monaliza	Santa	(Ozturk et al., 2011)

Likewise, a study on grain Cd accumulation in 35 wheat cultivars, grown in several field sites in Australia, revealed the meaningful impact of the cultivar as well as the site (Oliver et al., 1995) (Table 1). The observed genotypic variation uncovered the accumulating potential of the screened Australian cultivars. For example, low Cd accumulating cultivars, Egret and Oxley, were distinguished from cultivars in which Cd level exceeded the Australian maximum permissible concentration (MPC) of 0.05 mg Cd kg^{-1} fresh weight.

A similar study on New Zealand wheat cultivars also confirmed the genotypic inheritance of the Cd accumulating trait (Gray et al., 2001) (Table 1). Despite the wide soil Cd range (0.09-0.45 mg kg^{-1}) among the different sites, which was likely originated from application of phosphate fertilisers, the results denied any significant correlation with grain Cd content. The Cd level ranged about 4-fold among the thirty-three analysed cultivars. To reduce the grain Cd burden, they highly recommended site crop management.

Another research with the same purpose was carried out on 49 rice genotypes cultivated in two different soil types containing 0.3 to 10.4 mg Cd kg^{-1}. The results revealed a pronounced varietal difference in seed Cd accumulation (Arao and Ishikawa, 2006) (Table 1). Rice varietal difference in Cd accumulation was also reported in two previous studies (Arao and Ae, 2001; Liu et al., 2005) (Table 1). Thereby, some commercial cultivars including Koshihikari were categorised into the low Cd grain group, whereas several indica or indica-japonica cultivars such as Milyang23 and Peh-Kuh-Tsao-Tu were found to be suitable only for phytoremediation as they contained high Cd levels in their seeds and shoots. These cultivars showed a similar Cd uptake potential in both soil types. The other interesting finding from this study was a meaningful correlation between contents of Cd and Zn or Mn in the shoots suggesting the involvement of the same uptake and translocation mechanisms of these divalent ions.

The concept of cadmium safe cultivar (CSC) has been grounded on the basis of the observed genotypic variation in Cd uptake and accumulation among the different plant cultivars (Yu et al., 2006). In CSC, the edible parts accumulate Cd at levels low enough for safe consumption, even when such cultivars are grown in Cd-polluted soils. To evaluate this level, it is compared to the maximum permissible concentration (MPC) proposed by either national health authorities or international health organisation (WHO). The national and WHO MPC could be the same or different based on the differences between the average daily consumption of crops in that particular country and the world. For example, the Chinese standard Cd limit for rice set by the National Food Hygiene Standard of China (NFHSC) is the same as the limit proposed by WHO (0.2 mg Cd kg^{-1} F.W.) (Yu et al., 2006; Zhan et al., 2013). Conversely, for cabbage, the Chinese national and WHO MPCs differ greatly (NFHSC MPC=0.05 mg kg^{-1} F.W., WHO MPC=0.2 mg kg^{-1} F.W.) (Liu et al., 2010; Qiu et al., 2011). In such a case, it seems that consideration of national MPC would be more logical and secure, as consumption rates of crops can vary greatly among different countries.

Aside from MPC, some additional criteria have also been suggested for selection of CSCs (Chen et al., 2012; Li et al., 2012; Liu et al., 2010). For instance, Chen et al. (2012) considered a yield limit in selection of CSCs among 50 Chinese cabbage (*Brassica rapa*) cultivars. They believed that growing CSCs in Cd-polluted soils should not decrease their yields substantially. Enrichment factor (EF) and translocation factor (TF) were also calculated for these cultivars. Enrichment and translocation factors are two indices usually defined as the ratio of plant organ metal concentration to soil metal concentration, and the ratio of target organ (e.g. grain or tuber) metal concentration to origin organ (e.g. shoot or root) metal concentration, respectively. In the case of CSCs, these indices should be lower than one.

Based on these criteria, among 50 studied pakchoi cultivars, Chen et al. (2012) only found three cultivars including Hangzhouyoudonger, Aijiaoheiye 333, and Zaoshenghuajing that were qualified to be introduced as CSCs (Table 3).

Trial experiments are often run for about 3-4 months which is the average period of a growing season. The seed/seedlings are cultivated in the soils with or without artificial Cd spiking under field conditions. Alternatively, the plants can be grown hydroponically. Soil/solution Cd levels usually are not set higher than 10 mg Cd kg^{-1} as application of environmentally irrelevant or unrealistic high Cd levels will not lead to identification of CSC (Hongjiang et al., 2014; Liu et al., 2010; Yu et al., 2006; Zhang et al., 2013). For instance, treatment of 43 paddy rice (*Oryza sativa* L.) cultivars with 77 mg Cd kg^{-1} caused exceeding of their Cd contents from NFHSC (0.2 mg kg^{-1}). Even in cultivars identified previously as CSCs in a low Cd regime, Cd content was found up to 1.75 mg Cd kg^{-1} (Yu et al., 2006). The results on yield were in disagreement with the expected synergistic correlation between yield depression and grain Cd content as the yield rate in some high Cd cultivars was much higher than CSCs.

In the literature surveyed above, many works have been carried out to identify crop cultivars with low Cd accumulation, but only few of them attempted to evaluate these cultivars further to find whether they can be introduced as CSCs or not. Moreover, the previous studies mostly focused on few staple crops including wheat (*Triticum aestivum*) and rice (*Oryza sativa*), whereas other important crops such as potato comprising more than 50 percent of dietary Cd intake were neglected (Dunbar et al., 2003; Reid et al., 2003). The value of identification of high Cd crop cultivars should not be neglected as well. Since, exclusion of them for consumption purposes can noticeably reduce dietary Cd level, particularly, in countries with Cd-polluted soils.

2.2. Aims

There is a paucity of information about natural variation in Cd accumulating abilities of different potato cultivars grown in New Zealand. This is a pre-requisite to take advantage of this source of variation for identification of low Cd accumulating potato cultivars as a practical strategy to minimise dietary Cd intake through consuming potatoes. Furthermore, this approach can lead to identification of nutrient-enriched cultivars which can be used in biofortification programmes.

In recent years, the concept of cadmium safe cultivars (CSCs) has been proposed which is based on observed Cd uptake variation among cultivars (Liu et *a*l., 2010; Zhang et al., 2014). Since Cd accumulation is known to be affected by many factors, more studies are needed to evaluate the importance of this concept. In particular, there are relatively few investigations into crops grown in soils with a low level of soil Cd from different countries such as New Zealand which has comparatively few heavy manufacturing industry and mining. The main source of low levels of soil Cd in many parts of New Zealand including Canterbury is application of phosphate-based fertilisers contaminated with Cd (Chen et al., 2007; McDowell et al., 2013).

Hence, the present study aimed to:

(1) Evaluate the differences in tuber Cd accumulation of ten randomly selected potato cultivars grown in a field environment such as a seed production block with low soil Cd level in the Canterbury region of New Zealand which is away from heavy manufacturing industry, mining or intense agricultural production practices,

(2) Seek to identify any interactions among Cd and other elements including iron (Fe), zinc (Zn), manganese (Mn), copper (Cu), and calcium (Ca) considered to be nutrients in potato grown in this environment, and thus more desirable in the human diet (Chen *et al*., 2007).

2.3. Materials and Methods

2.3.1. Plant materials and soil type

Over 30 potato cultivars were grown outdoors for seed production in a block situated approximately one kilometre east of Lincoln, Canterbury within the 2011-2012 growing season. The soil type was Wakanui (silt loam on sandy loam) with average pH 5.9 and Olsen phosphate value of 18 ppm. The applied fertiliser was Cropmaster 15 (250 kg/ha, pre-planting application) containing 10% phosphorus with lower than 280 mg Cd kg^{-1} and no irrigation was applied. The cultivars were grown in plots (size ranged from 15 to 50 plants). For this study, ten potato cultivars with a broad range of flesh colours (Fig. 2) were randomly selected including Red Rascal, Russet Burbank, Fianna, Agria, Laura, Purple Heart, Purple Passion, Yukon Gold, Moonlight and Summer Delight. Six typical plants (similar development, growth vigour and disease-free) of each of the selected cultivars were chosen and at least two tubers were selected randomly from each of the six plants. The tubers as well as the soil samples immediately surrounding the tubers were harvested in mid-Feb 2012 and stored in a cold room at $4^{o}C$ until trace element analysis at a later date.

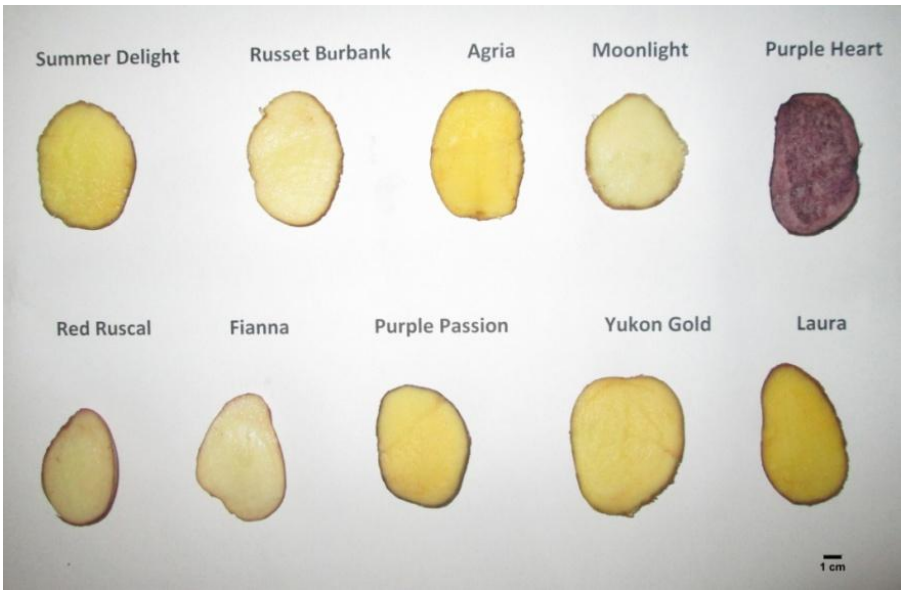


Figure 2. Flesh colours of ten potato cultivars in this study

2.3.2. Soil trace element analysis

Soil samples were oven dried at 30°C for 6 days followed by grinding and sieving through a one mm stainless steel mesh. Dry material (~1.0 g) of each sample was then digested in a mixture of 50% nitric acid (HNO_3) (4 mL) and 20% hydrochloric acid (HCl) (10 mL) by refluxing (~85°C) for one hour. Afterwards, clear digests were allowed to cool at room temperature and then made up to 20 mL with milli-Q water. Subsequently, 0.5 ml of each digest was diluted by addition of 10 mL 2% HNO_3 (Gaw et al., 2006). The concentrations of elements including Cd, Fe, Zn, Cu, Mn, and Ca in the digest solutions were determined using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) *(*Agilent 7500 cx). A certified reference material (Inorganics in Marine Sediment, NIST SRM 2702), procedural blank and duplicates (one per 10 samples) were analysed with each batch of samples. Recoveries for the certified reference material were acceptable ranging from 86 to 108 %.

2.3.3. Tuber trace element analysis

The tubers were rinsed with milli-Q water and peeled carefully. Afterwards, longitudinal samples were taken from the centre of tubers by a cork borer (15 mm diameter). The samples were freeze-dried for 10 days and then milled to fine powders. Subsequently, 0.5 g of each sample was stood with 5 mL of 65% HNO_3 for an hour before digestion using the method described above. They were allowed to cool at room temperature and then their volumes adjusted to 20 mL with milli-Q water. Afterwards, 1 ml of each digest was diluted five-fold by addition of 4 ml 2% HNO3. The concentrations of trace elements were detected by ICP-MS. A certified reference material (rice flour, NIST SRM 1568a), a procedural blank and duplicates (one per 10 samples) were involved in each batch of samples. Recoveries for the certified reference material were acceptable ranging from 84 to 107 %.

2.3.4. Enrichment factor

To evaluate the abilities of these ten cultivars to accumulate Cd in the tuber from the soils, an enrichment factor (EF) was calculated. This is an index of soil-plant transfer calculated as the ratio of the metal concentration in the plant organ to the metal concentration in the soil (Olowoyo et al., 2010).

2.3.5. Statistical analysis

For the soil trace element analysis, thirty samples were taken from different parts of the field and tuber analysis was carried out with six independent replicates (tuber cores) for each cultivar. Analysis of variance (ANOVA) of the data obtained was carried out using the SPSS software (Version 19.0). Duncan's test was used to determine significant differences between the means ($p<0.05$) and correlations were assessed by Pearson's correlation test.

2.4. Results

2.4.1. Soil trace element analysis

Cadmium concentration in the soil samples taken from the potato growing field ranged from 0.04 to 0.08 mg kg^{-1} dry weight (DW) (Table 4) with the average value of 0.06 mg kg^{-1}. Similar to the range of soil Cd, the minimum and maximum concentrations of Fe, Zn, Cu, Mn and Ca in the potato growing field were about or within 2-fold difference (Table 4).

Table 4. Concentrations of Cd and five other elements in the soils (0-20 cm depth) collected from the potato growing field in the present study.

Element	Median	Min.	Max.
	—— (mg kg^{-1} DW) ——		
Cd	0.05	0.04	0.08
Cu	1.44	1.14	2.11
Zn	17.28	14.10	34.74
Mn	59.21	48.26	76.01
Fe	515.66	402.78	615.95
Ca	1170.85	1054.93	2016.24

2.4.2. Tuber trace element analysis

Tuber Cd concentration varied from 0.04 to 0.34 mg kg^{-1} DW among the ten potato cultivars (Table 5) with median and mean values of 0.13 and 0.14 mg kg^{-1} DW. Compared with data from other countries, these levels are at the higher end of the range and similar to Cd content of cultivars cultured in Belgium (Table 5).

Table 5. Cd contents of potato tubers in the studies from different countries

Country	Number of Samples	Median	Min.	Max.	Reference
		(mg Cd kg^{-1} DW)			
Australia	359	0.16	0.02	1.16	(McLaughlin et al., 1997)
Belgium	108	0.12	0.03	0.34	(Oporto et al., 2007)
Canada	75	0.11	0.04	0.20	Fan *et al.* (2009)
Switzerland	101	0.06	0.01	0.22	(McLaughlin et al., 1997)
Sweden	75	0.04	<0.01	0.14	(Oporto et al., 2007)
Norway	79	0.06	<0.01	0.22	(Oporto et al., 2007)
Poland	90	0.05	<0.01	0.45	(McLaughlin et al., 1997)
New Zealand	60	0.13	0.04	0.34	Our study

Among the ten potato varieties investigated in the present study, three groups exhibiting different trends in tuber Cd accumulation could be distinguished (Fig. 3). Laura, Yukon Gold and Purple Passion were potato varieties showing the highest levels of Cd accumulation with Cd concentrations of 0.18-0.21 mg kg^{-1} DW. Conversely, Summer Delight was the cultivar accumulating the least tuber Cd (range of 0.03-0.07 mg kg^{-1} DW and mean Cd content of 0.05 mg kg^{-1}). The other cultivars (Fianna, Red Rascal, Purple Heart, Moonlight, Agria and Russet Burbank) had moderate tuber Cd contents of around 0.10 - 0.13 mg kg^{-1} which were well-below the MPC (Fig. 3).

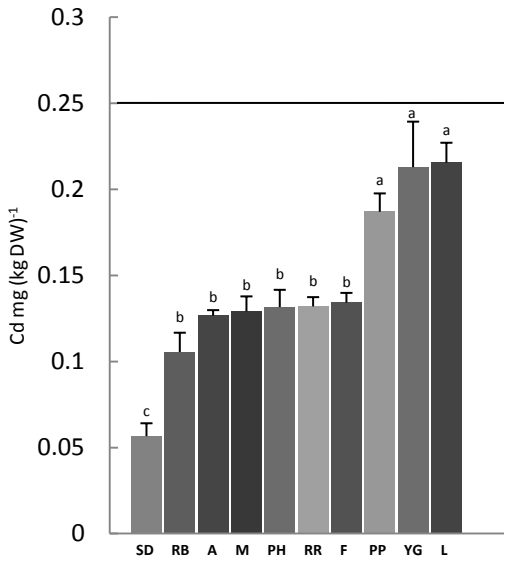


Figure 3. Cadmium concentration in the ten potato cultivars: Summer Delight (SD), Russet Burbank (RB), Agria (A), Moonlight (M), Purple Heart (PH), Red Rascal (RR), Fianna (F), Purple Passion (PP), Yukon Gold (YG) and Laura (L). The concentrations are means ± SE (n=6) shown as mg kg^{-1} DW. The vertical bars show standard errors and the horizontal line represents the MPC.

2.4.3. Enrichment factor

According to the obtained results presented in Fig. 4, only Summer Delight had an EF less than 1 (0.95) while the enrichment factors of the other nine cultivars were well above 1.

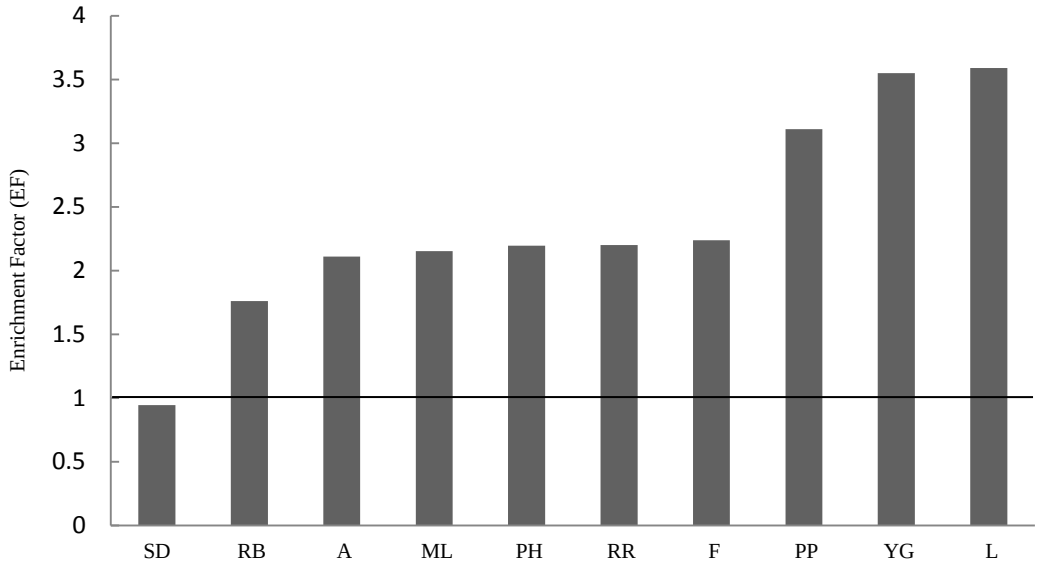


Figure 4. Enrichment factor (EF) of cadmium in the tubers of ten potato cultivars: Summer Delight (SD), Russet Burbank (RB), Agria (A), Moonlight (M), Purple Heart (PH), Red Rascal (RR), Fianna (F), Purple Passion (PP), Yukon Gold (YG) and Laura (L). Each bar represents the ratio of the mean Cd concentration in the tubers of individual cultivar to the mean Cd concentration in the soil.

2.4.4. Correlation between uptake of Cd and other elements

Summer Delight with an average of 31 mg Fe kg^{-1} DW demonstrated a very strong accumulating ability for Fe compared with the other nine cultivars (Fig. 5A). The other cultivars (with the exception of Moonlight and Fianna that contained moderate Fe concentrations of 16 and 18 mg kg^{-1}), showed accumulation of Fe in the range of 21 to 24 mg kg^{-1} (Fig. 5A). All the cultivars studied here had moderate Zn contents ranging from 12 mg kg^{-1} in Moonlight and Fianna to 21 mg kg^{-1} in Purple Heart (Fig. 5B). The contents of Cu (3.5-6.5 mg kg^{-1}) and Mn (4.0 -7.7 mg kg^{-1}) in the tubers also showed variation among cultivars even though they were low relative to other mineral levels (Fig. 5C&D).

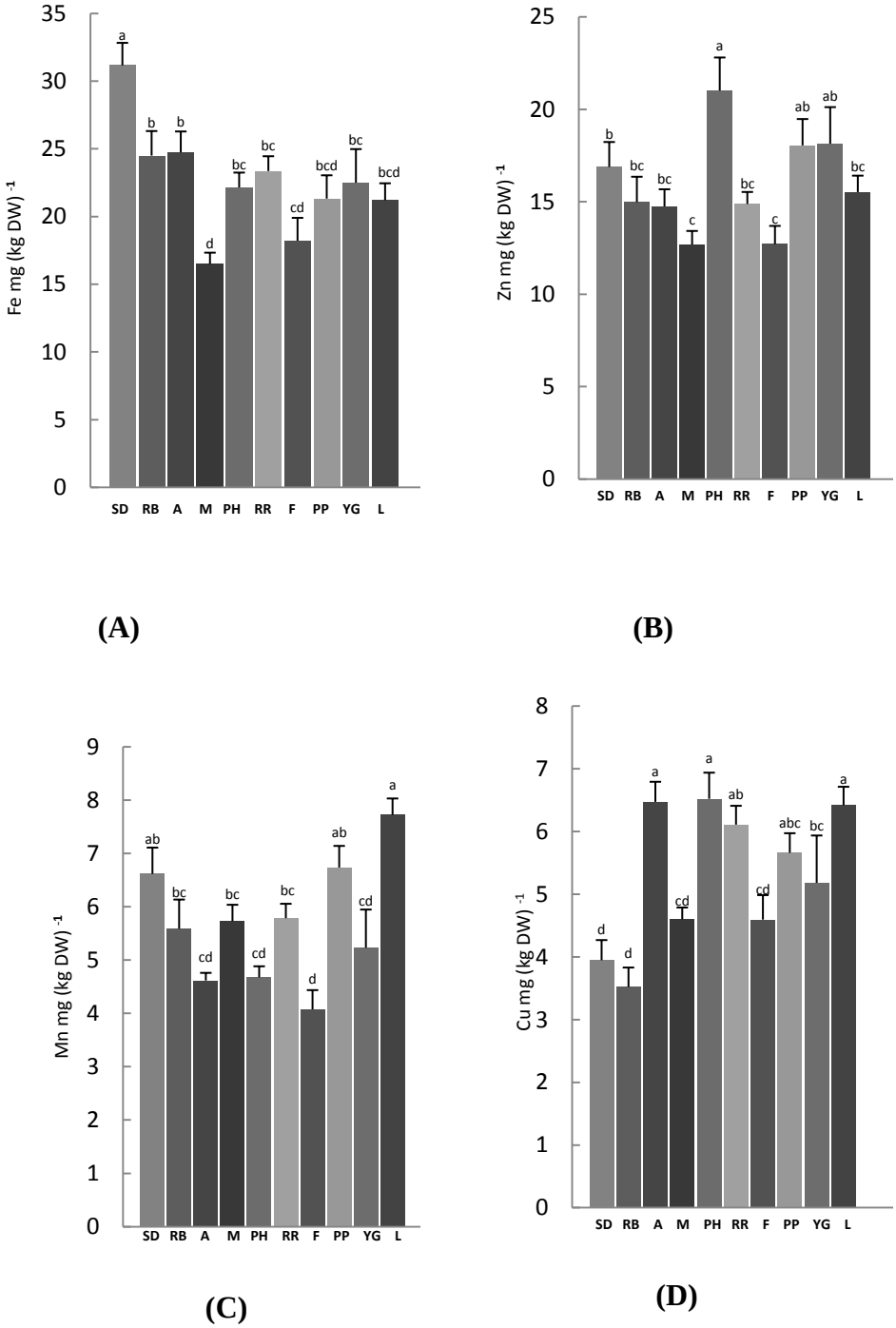


Figure 5. Concentrations of (A) iron, (B) zinc, (C) manganese and (D) copper in the ten potato cultivars: Summer Delight (SD), Russet Burbank (RB), Agria (A), Moonlight (M), Purple Heart (PH), Red Rascal (RR), Fianna (F), Purple Passion (PP), Yukon Gold (YG) and Laura (L). The concentrations are means ± SE (n=6) shown as mg kg^{-1} DW. The vertical bars show standard errors.

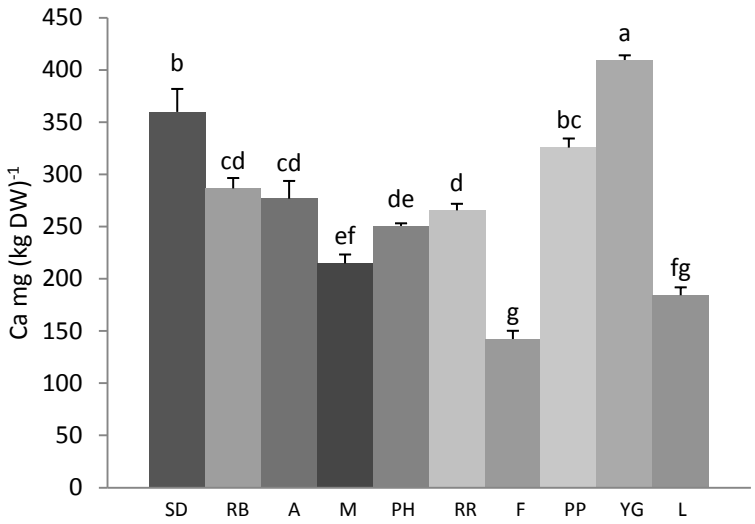


Figure 6. Concentration of calcium (Ca) in the ten potato cultivars: Summer Delight (SD), Russet Burbank (RB), Agria (A), Moonlight (M), Purple Heart (PH), Red Rascal (RR), Fianna (F), Purple Passion (PP), Yukon Gold (YG) and Laura (L). The concentrations are means ± SE (n=6) shown as mg kg^{-1} DW. The vertical bars show standard errors.

Summer Delight was also the most Ca-enriched cultivar with 360 mg kg^{-1} mean just after Yukon Gold contained 409 mg Cd kg^{-1} (Fig. 6). This concentration is comparable with that of Australian cultivar: Kennebec (370 mg kg^{-1}). On the other hand, F, L and M accumulated the lowest Ca concentrations (142, 184 and 214 mg kg^{-1}) respectively, which were well-below that of low Ca Australian cultivar: Wilwash (238 mg kg^{-1}).

Correlation analysis revealed that there was a relationship between Cd accumulation and the accumulation of other trace elements. For instance, significant positive relationships between Cd and Zn, Cu, or Mn were observed in the cultivars studied (Table 6). On the other hand, Fe and Ca accumulations had no significant relationship with Cd accumulation, whereas they were negatively correlated to each other.

Table 6. Correlation among accumulation of Cd and Fe, Zn, Cu, Mn and Ca in the ten potato cultivars used in the present study.

	Cd	Fe	Zn	Cu	Mn	Ca
Cd	1.000	-0.088	0.400**	0.582**	0.357**	0.030
Fe		1.000	0.630**	0.269*	0.404**	-0.250 *
Zn			1.000	0.605**	0.331**	-0.205
Cu				1.000	0.268*	-0.018
Mn					1.000	-0.172
Ca						1.000

*, **: Correlation is significant at 0.05 and 0.01 levels respectively (n=10).

2.5. Discussion

Only few studies were carried out to probe the extent of variation in Cd bioaccumulation in tubers of different potato varieties grown in non-Cd contaminated soils with different trace elements, although this line of investigations is important to obtain more comprehensive insights about soil Cd uptake into the edible plant parts. In the present study, the Cd levels in the potato growing field for seed production were low compared to the known Cd levels in the agricultural soils of Canterbury and New Zealand, with average values of 0.17 mg kg^{-1} and 0.35 mg kg^{-1} reported, respectively. Therefore, the soil samples in the present study were not considered to be Cd contaminated as their average Cd content was less than one tenth of the New Zealand guideline (1 mg kg^{-1}) (Kim, 2005; Taylor, 2007). This result was similar to the average Cd concentration of five experimental sites in Quebec (0.03-0.07 mg Cd kg^{-1} DW) used for the same purpose (to evaluate Cd accumulating potential of potato tubers) (Fan et al., 2009). In contrast, the soil levels of Fe, Zn, and Mn were 3-6 fold higher in our study than the study in Quebec, Canada (Fan et al., 2009).

The average Cd levels found in potato tubers in this study was same as that of potatoes in New Zealand diet reported in 2009 showing an increasing trend compared with two previous reports in 2003/2004 and 2000 (to transform fresh weights to dry weights, dry matter concentration was assumed 20%) (Fan et al., 2009; Ministry of Agriculture and Foresty, 2009; Oporto et al., 2007). Clearly, no Cd levels were found to exceed the MPC (0.25 mg kg^{-1} DW) for potato tuber as proposed by WHO/FAO (Fan et al., 2009). They were also well-below the Australia-New Zealand standard limit for tuber vegetables which is 0.5 mg Cd kg^{-1} DW (the fresh weights were transformed to the dry weights as described above) (FSANZ, 2011). However, it seems that more caution is needed in

cultivation of the cultivars Laura, Yukon Gold and Purple Passion as 10 percent of their samples exceeded the MPC, despite the soil Cd content being around 10% that of the national average. If Cd uptake is not saturated at these low soil levels then it would be expected that higher Cd soil contents would thus lead to higher Cd accumulation levels that would be likely to exceed the MPC. Therefore, growing these cultivars in soils with higher Cd contents may pose a concern to food safety.

The ten cultivars studied here showed a significant variation ($p<0.05$) in tuber Cd content. This is in agreement with a study on two Australian cultivars, Kennebec and Wilwash, which were classified as high and low accumulator cultivars with averages of 0.24 and 0.15 mg Cd kg^{-1} DW, respectively (Dunbar et al., 2003). Only Summer Delight had an EF less than 1 (0.95; Fig. 4), suggesting that it may be a Cd indicator cultivar (reflecting Cd content of soil) (Ahmad et al., 2007; Olowoyo et al., 2012). Cadmium safe cultivars (CSCs) are described as cultivars with EF<1 and edible parts containing Cd concentrations lower than the MPC (Chen et al., 2012). Hence, Summer Delight is a candidate to be promoted as a cadmium safe cultivar in New Zealand. The enrichment factors of the other cultivars were well above 1 (Fig. 4), depicting them as Cd enriched cultivars (accumulators) (Olowoyo et al., 2012). The Yukon Gold, Laura and Purple Passion cultivars all exhibited EF's in excess of 3, suggesting that these cultivars are particularly effective Cd accumulators.

Deficiency of essential elements including Fe, Zn, Cu and Mn in plants can affect the quantity and quality of human diet (Grotz and Guerinot, 2006) . It is also interesting that Summer Delight exhibited a strong Fe accumulating ability (an average of 31 mg Fe kg^{-1} DW) compared to the other cultivars studied here as well as with two Australian cultivars (Wilwash and Kennebec), which contained 10 and 20 mg Fe kg^{-1}, respectively (Dunbar et al., 2003). In the literature, the range of tuber Zn concentrations has been reported to vary from 2

to 37 mg kg^{-1} (Haynes et al., 2012). Hence, the tuber Zn values found in the present study were not exceptional compared to the previously reported levels. The Cu and Mn levels in the varieties studied here, were, however, about half of those in the nine potato cultivars grown in the different locations of USA (True et al., 1978).

Summer Delight, with the lowest Cd and highest Fe content, is of particular interest as it could not only be considered as a cadmium safe cultivar but also as a Fe-enriched tuber vegetable. It also showed a moderately high accumulating potential for two other essential trace elements: Zn and Mn as well as Ca which is the most important macronutrient. As potatoes are characteristically low in Ca (Reid et al., 2003; Subramanian et al., 2011), this can really highlight the value of this cultivar. Hence, Summer Delight with low Cd and high nutritional value can be applied as a desirable potato cultivar for either consumption or development of a biofortified cultivar with enhanced nutrients for improved plant growth (Haynes *et al*., 2012).

The pronounced difference in tuber Cd contents of these ten cultivars, varying about 2-4 fold, can be attributed to qualitative and/or quantitative differences in Cd uptake and/or partitioning (Dunbar et al., 2003; Grant et al., 2008). The significant correlations between the trace element concentrations in the cultivars suggest the involvement of common metal transporters, either for uptake from soils or translocation within the plant. The relationship between Cd accumulation and the accumulation of other trace elements, revealed by the correlation analysis, has been well-documented in the literature. In particular, uptake of Cd and Zn has been shown to be correlated as they are chemically very similar (they are neighbours in group IIb periodic table) (Chen et al., 2007; Smith and Brennan, 1983). In fact, one of the main reasons can be the presence of same metal transporters for two or more divalent metals. The main transporter families in plants are heavy metal ATPases (HMAs), Nramps,

Cation Diffusion Facilitator (CDF) and ZIP acting at either tissue level (e.g. mesophyll cells to epidermal cells) or organ level (e.g. the roots to shoots) (Hall and Williams, 2003; Ovečka and Takáč, 2014). On the other hand, based on correlation between Ca and Fe, other possible mechanisms might be involved including direct uptake across the periderm. However, as we only analysed Cd contents of the tubers and did not analyse the leaves and roots, the presented data set does not allow a comprehensive assessment regarding the mechanisms associated with the observed variation. Therefore, further studies on the other potato organs including the root and shoot with more samples and cultivars are warranted and could give a more comprehensive picture of variation in Cd accumulation. As other factors including soil properties, fertiliser level and weather condition can affect Cd uptake in plants, evaluation of their impacts is also highly recommended (Larsson Jönsson and Asp, 2011).

Chapter 3- Investigations to Generate Potato Cell Lines and Plant Regenerants with Increased Cadmium Resistance

3.1. Introduction

It is well-documented that variation in HM resistance can occur naturally among different plant cultivars (Grant et al., 2008). This may not be broad enough for the resistant cultivars to be found. Moreover, if HM resistance is sought in particular genotypes, this approach is not applicable obviously. Thus, to improve plant HM resistance, there is often a need for employing a breeding method such as conventional plant breeding methods. However, due to the lengthy procedures and high costs of traditional breeding practices, plant breeders seldom give the HM resistance trait as a top priority compared to the traits that are known to affect yield such as those associated with resistance to drought, salinity or diseases. *In vitro* breeding using plant tissue culture techniques can be a promising alternative, but so far it has been mostly exploited to develop resistant plants to other abiotic stresses including drought and salinity (Rai et al., 2011). Here, the methodologies used in the studies carried out on *in vitro* breeding of HM-resistant plants to date have been summarised.

In vitro breeding basically starts with explants removed from mother plants grown under *in vitro* conditions (Fig. 7). Fully-developed plant structures including the leaves and shoots have been used as the initial explants cultured on a common basal plant tissue culture medium such as Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), solidified with agar and supplemented with different combinations and concentrations of plant growth regulators (PGRs). The two principal PGRs often used are auxins and cytokinins, which at optimised concentrations, can trigger totipotent cells to

form an unorganised tissue called callus comprising of undifferentiated parenchyma cells (Gaspar et al., 1996).

Callus initiation often starts from edges or wounded parts of explant tissues, and then can gradually cover whole of the explant surfaces. The appropriate culture conditions such as optimised temperature and lighting are the other requirements provided in a control chamber or growth room. Plant species and genotype have been found to affect the time and frequency of callus induction greatly (Sharma and Agrawal, 2005).

Callus cells are commonly proliferated on an agar-solidified medium containing the same combinations and concentrations of phytohormones as the initiation medium. Otherwise, less often they can be used to form suspension culture in liquid medium. At an early stage of callus initiation or proliferation, the chance of somaclonal variation occurrence is very high (Wang and Wang, 2012). It was found advantageous to add a HM of interest in the culture medium at this stage to help to select for any HM-resistant somaclonal variant cells. However, HM exposure at the callus induction stage has rarely been applied in many other studies. The callus proliferation stage normally takes from a few weeks to a few months. Prolonged subculture with stepwise increases in the concentrations of HMs could also help to capture the chance of somaclonal variation occurrence. The limitation of this approach is that the regenerating ability of calli might be adversely affected (Bairu et al., 2011; Kaeppler et al., 2000). As the callus cells are not homogeneous, different exposure periods and levels may trigger different responses from the different constituent cell populations in the proliferating callus culture. Some phytohormonal treatments triggered callus cells to undergo embryogenesis instead of proliferation, and the embryos formed can also exhibit HM resistance (Von Arnold et al., 2002).

Calli can be exposed to the stress factors or selecting agents (HMs) during proliferation (subculture) or even from earlier (the callus induction stage) to select for the desirable trait (HM-resistance). Exposure of callus to a sub-lethal concentration of HM has been applied more than stepwise increases in HMs in the previous studies. One of the reasons for this could be to minimise the chance of epigenetic changes which could be inherited meiotically and obscure the selection process of true resistant plants during prolonged subcultures (Rai et al., 2011). The second reason is to avoid the loss of regeneration ability which is the other obstacle for *in vitro* approach and is more likely to be associated with the approach involving lengthy stepwise increases in HMs to select for HM-resistant calli (Rai et al., 2011). Nevertheless, a prolonged selection procedure (4-8 months) was applied in more than one fourth of the previous studies resulting in reduced shoot regeneration to less than 10 percent (Van Sint Jan et al., 1997).

Ultimately, to regenerate plantlets from HM-treated calli or embryos, they are transferred to plant regeneration media often lacking HMs (Fig. 7). These media usually contain gibberellic acid (GA_3) which is known as a key germination promoter (Gaspar et al., 1996). Although HMs at high concentrations are likely to inhibit plant regeneration, at low concentrations HMs themselves are also known as morphogenesis stimulators as they are able to inhibit ethylene synthesis (Roustan et al., 1989; Rout et al., 1998). Compared to the mother plants cultured under *in vitro* conditions or plants regenerated from control callus not exposed to HMs, the regenerates from HM-treated calli may show more, less or same HM resistance (Fig. 7).

Many factors such as polyploidy (having multiple sets of chromosomes) and plant regeneration potential can affect the progress of *in vitro* breeding. Potato (*Solanum tuberosum* L.) is a good example as it is a tetraploid species (4n=48) which can be readily regenerated from single cells and callus (Wilson et al.,

2009). Among the numerous potato cultivars, "Iwa" is one of the cultivars most amenable for application of *in vitro* selection due to its high efficiency of *in vitro* shoot regeneration (Wilson et al., 2010). In a study on resistance of potatoes to common scab disease, Iwa showed significant higher levels of resistance compared to Russet Burbank which is a commercial cultivar. Although Iwa is no longer widely cultivated, it has been suggested as a model system in potato studies (Wilson et al., 2010). All these suggest Iwa as a promising cultivar for improving potato resistance to abiotic stresses including HM stress.

It is necessary for *in vitro* plant breeders to set individual *in vitro* selection programme for each genotype because of the varietal differences in cell regeneration potential and HM resistance. This usually involves optimisation of combinations and concentrations of phytohormones required for callus induction, proliferation and organogenesis. Also the selective agent (HM) level must be carefully chosen. Application of 1-naphthalene acetic acid (NAA) and 6-benzyladenine (BA) is well-documented for callus induction in many potato cultivars (Hansen et al., 1999; JayaSree et al., 2001; Khatun et al., 2003). Supplementation of picloram (PIC) in the culture medium as the only PGR was also found to result in callogenesis from tubers of seven potato cultivars (Hagen et al., 1990). Callus induction medium is often sufficient for callus proliferation or subculture and it is often not necessary to optimise a separate callus proliferation medium.

For Iwa potato, a protocol for callus induction from stem internodes and its subsequent regeneration protocol have been described by Wilson et al. (2009). In this study, calli were initiated on agar-solidified MS medium supplemented with 1.7 μM NAA, 14.4 μM gibberellic acid (GA_3), and 8.8 μM BA. The regenerants were successfully obtained on the medium with the same composition and concentrations of PGRs except BA substituted with 4.56 μM

zeatin. To the best of my knowledge, no tissue culture work has been done on *in vitro* breeding of any potato cultivar for resistance to HMs.

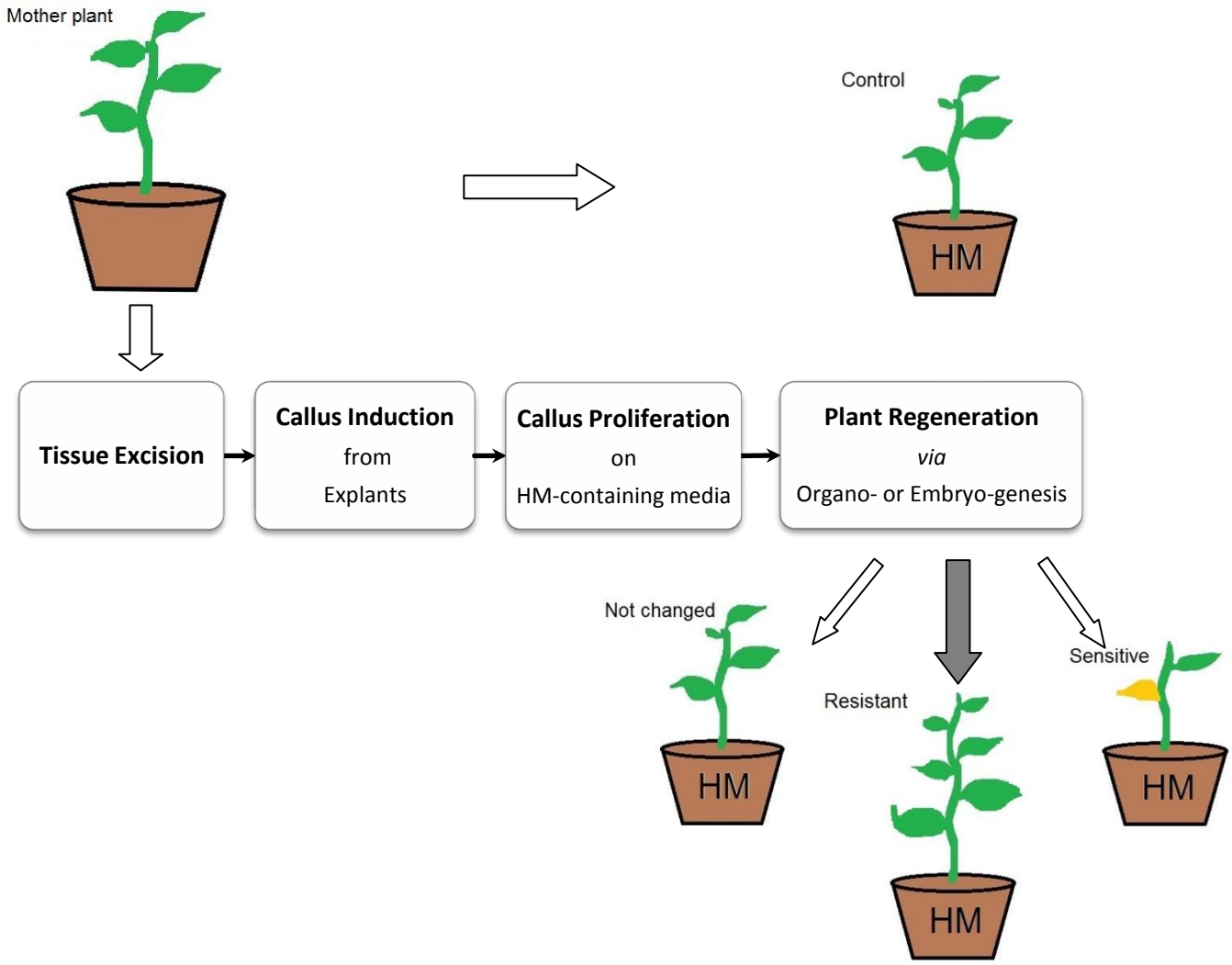


Figure 7. Schematic procedure of *in vitro* development of HM-resistant plants. After regeneration, the HM resistance level of the regenerants can be assessed by comparing with control plants derived directly from mother plants.

3.2. Aims

In vitro tissue culture inducing somaclonal variation was chosen to promote and select potato variants with improved Cd resistance. In order to achieve this aim, the following objectives were set for this chapter.

- Development of optimised protocols for callus induction and growth
- Determination of callus sensitivity to Cd stress to identify the sub-lethal Cd concentration required for selection of Cd resistant somaclones
- Establishment of optimised protocols for organogenesis

3.3. Materials and methods

3.3.1. Plant materials and culture conditions

Potato (*Solanum tuberosum)* cultivar Iwa was chosen for the present study, based on its characteristics mentioned before (3.1). The source of potato plantlets was from the stock plants that were used in a previous study on microtuber induction from the axillary buds under aseptic conditions (Yoon and Leung, 2004). The plantlets were cloned regularly (by 6-8 week interval) through *in vitro* culture of 3-cm long nodal segments in half-strength MS basal medium (Murashige and Skoog, 1962) (Appendix).

All the media were supplemented with 3% (w/v) sucrose, adjusted to pH 5.8 with 1M KOH or HCL, and solidified with 0.8% (w/v) agar. Afterwards, they were autoclaved at 121^{o}C under 103 kPa for 20 minutes. To sterilise tissue culture tools including scalpels, forcepses and scissors, these were first surface wiped with 70% ethanol and then dipped in a hot glass bead dry steriliser (Steri 250, Switzerland) for 20-25 seconds. All the plantlets were grown inside plastic tissue culture jars (8.5 cm height and 6.5 cm diameter) in a growth room at 21$\pm$1^{o}C and under continuous lighting (Sylvania Gro-Lux lamps 36W*)*. The described sterilisation procedure and culture conditions were used in all the experiments hereafter as well, unless otherwise mentioned.

3.3.2. Determination of sensitivity of plantlets to cadmium stress

To assess sensitivity of Iwa potato plantlets to Cd, shoot tips (with approximately same diameter) carrying three leaves were isolated from well-grown micropropagated plantlets and cultured in half-strength basal MS medium containing 0 (control), 27, 54, 109, 218 and 436 μM cadmium chloride ($CdCl_2$). Each treatment was carried out with three replicates, each containing 40 shoots. All the cultures were kept in a growth room at 21 ± 1°C with continuous lighting. Afterwards, shoot and root lengths (by days 9 to 18), and leaf number were determined and Cd injury symptoms including chlorosis, necrosis and leaf curling (by days 12 to 22) were evaluated based on visual inspection. This experiment was designed based on the similar work on the other potato cultivar (Premiere 1) assessing the effects of cadmium on shoot culture growth (Domazlicka and Opatrny, 1989). The lowest lethal Cd concentration (which can completely inhibit the plantlet growth) identified here, was also used in Chapter four as a primary screen for tissue culture-derived plant lines.

3.3.3. Callus induction

Fully-developed leaves (with 2 cm width) and internodal segments (with 2 cm length and 0.5 cm diameter) were excised from well-grown plantlets (Fig. 9). To increase the frequency of callogenesis from the leaf explants, the tips of the leaves were removed (Ikeuchi et al., 2013). Subsequently, the explants were cultured on agar-solidified half-strength basal MS medium (in plastic Petri dishes, 9 cm diameter) supplemented with:

- 1.7 μM NAA+14.4 μM GA_3+8.8 μM BA (the protocol established by Wilson et al. (2009)

- Different concentrations (8.28, 10.35, 12.42, 16.56 μM) of picloram (PIC) or NAA (5.37, 8.05, 10.74, 13.42 μM) singly or in combination with 2.21, 4.43, 6.65, and 8.87 μM BA (Table 9).

After placing the explants on the surface of media in the Petri dishes, strips of parafilm were used to seal around the Petri dishes. All these actions were carried out inside a Laminar Air Flow Cabinet (CLYDE APAC-HWS) and all the cultures were kept in a growth room at 21 ± 1°C with continuous lighting. There were 40 explants in each of the three replicates of a treatment. To determine the frequency of callus formation in each treatment, the number of explants forming callus was scored after 4 weeks of culture.

3.3.4. Callus proliferation

After three weeks of culture on the media containing 12.42 μM PIC or 5.37 μM NAA + 4.43 μM BA, the calli were carefully isolated from the original explants (internode or leaf) and cut into the two halves by a sharp scalpel. Afterwards, subdivided calli were sub-cultured on the same media on which they were initiated. The interval between two sub-cultures was two weeks. There were 40 pieces of calli in each of the three replicates of a treatment. All the cultures were kept in a growth room at 21 ± 1°C with continuous lighting.

To investigate the impacts of different lighting conditions on callus proliferation, calli were sub-cultured in the dark or under a 16/8 photoperiod. For the dark treatment, cultures were completely wrapped with aluminium foils to exclude light in the growth room used for most experiments in this thesis, and for the experiment under a 16/8 photoperiod the callus cultures were kept in another growth room. Callus proliferation rate was determined using the following equation (1):

$$(1) = \frac{\text{fresh weight of callus after prolifiration on an appropriate medium for 2 weeks}}{\text{fresh weight of callus before start of culture}}$$

3.3.5. Determination of callus sensitivity to cadmium

Determination of Cd sensitivity of callus culture is very critical for identifying the sub-lethal Cd level required for *in vitro* selection. Due to the lack of this knowledge about response of potato callus to Cd exposure, varying Cd levels used for treatment of potato plantlets (see section 3.3.2), were applied for determination of Cd sensitivity of potato callus during initiation and proliferation stages. Therefore, explants/fully grown calli (those that had undergone two rounds of sub-culture) were placed in plastic Petri dishes (9 cm diameter) containing the respective induction/proliferation media supplemented with 0 (control), 27, 54, 109, 218 and 436 μM $CdCl_2$. There were three replicates, each containing six explants or eight pieces of calli. Subsequently, callus induction frequency, morphology, growth rate and dry weight were evaluated in each treatment. To probe Cd resistance mechanism(s) involved, the changes in hydrogen peroxide (H_2O_2) content as well as activities of key antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (GPX) were assessed (Fig. 8).

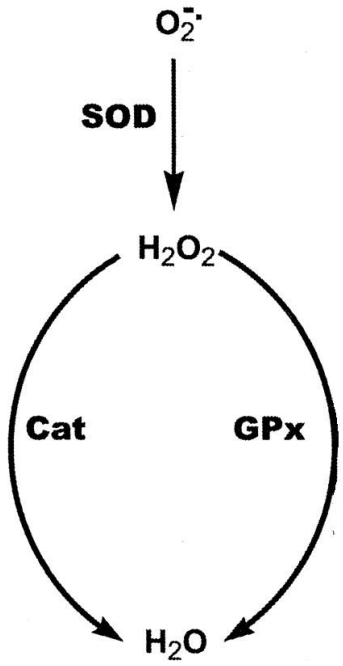


Figure 8. The main antioxidative defence mechanism involving three key enzymes: superoxide dismutase (SOD), catalase (CAT) and guaiacol peroxidase (GPx) in the callus cells (Hoen et al., 2003).

3.3.5.1. Callus formation frequency

This was evaluated by recording the presence or absence of callus cells after four weeks of culturing explants on callus induction media containing different concentrations of $CdCl_2$ (0 - 436 μM). The explants were also inspected visually for Cd injury symptoms including necrosis.

3.3.5.2. Callus morphology

The impacts of different Cd levels on well-grown calli (undergone two rounds of sub-culture) were investigated by inspecting them after two weeks of culture on the proliferation media containing 0, 27, 54, 109, 218 and 436 μM $CdCl_2$. Any sign of necrosis or colour change was recorded for each treatment.

3.3.5.3. Determination of relative growth rate

The fresh weights of calli that had undergone two rounds of sub-culture were accurately measured before and after two weeks of culture on the proliferation media containing one of the Cd concentrations used except 218 and 436 μM which were found to be lethal. Based on these data, relative growth rate (RGR) was calculated using the following formula (2).

$$(2) = \frac{\text{fresh weight of calli after culture on media supplemented with different concentrations of Cd - fresh weight of calli before start of culture}}{\text{fresh weight of calli before start of culture}} \times 100$$

3.3.5.4. Determination of dry weights

To evaluate the effects of non-lethal Cd concentrations (0, 27, 54, 109 μM Cd) on the callus water content, 100 mg fresh weight of calli from each treatment were dried in an oven (CONTHERM – CON8050) at 80°C for 48 hours to achieve constant weights. Afterwards, their weights were accurately measured and then water contents were calculated.

3.3.5.5. Hydrogen peroxide

Hydrogen peroxide (H_2O_2) level was determined according to Singh et al. (2006). Briefly, 0.5 g fresh weight calli were ground in liquid nitrogen using a mini pestle and mortar and then homogenised in 5 ml of ice-cold 0.1 % (w/v) tri chloro acetic acid (TCA). The homogenate was centrifuged at 12,000 g for 15 min at 4^{o}C. Afterwards, 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7), and 1 mL of 1 mM *potassium* iodide

(KI). Hydrogen peroxide content was measured by reading the supernatant absorbance at 390 nm using a spectrophotometer (BIO RAD-Smartspec^{TM} Plus).

3.3.5.6. Extraction of antioxidative enzymes

Sample extraction was carried out according to a modified Martins et al. (2014) method. One gram (fresh weight) calli were ground in liquid nitrogen using a mini pestle and mortar and homogenised in one ml of 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM Na_2EDTA, and 2% (w/w) insoluble polyvinyl polypyrrolidone (PVP). The homogenate was then centrifuged at 12,000 g for 15 min at 4^{o}C. Afterwards, the supernatant was concentrated using VIVASPIN 500 (5000 MWCO) ultrafiltration units. The activities of antioxidative enzymes were determined according to the following procedures.

3.3.5.7. Superoxide dismutase (SOD)

Superoxide dismutase (EC 1.15.1.1) activity was assayed according to the method of Martins et al. (2014) by measuring an increase in absorbance at 550 nm for 2 min in 100 mM potassium phosphate buffer (pH 7.6) containing 0.05 mM ferricytochrome-C, 0.5 mM xanthine, 0.1 mM EDTA and xanthine oxidase. The enzyme activity was determined as the enzyme quantity required to inhibit the reduction of ferricytochrome-C by 50 percent, per min and mL.

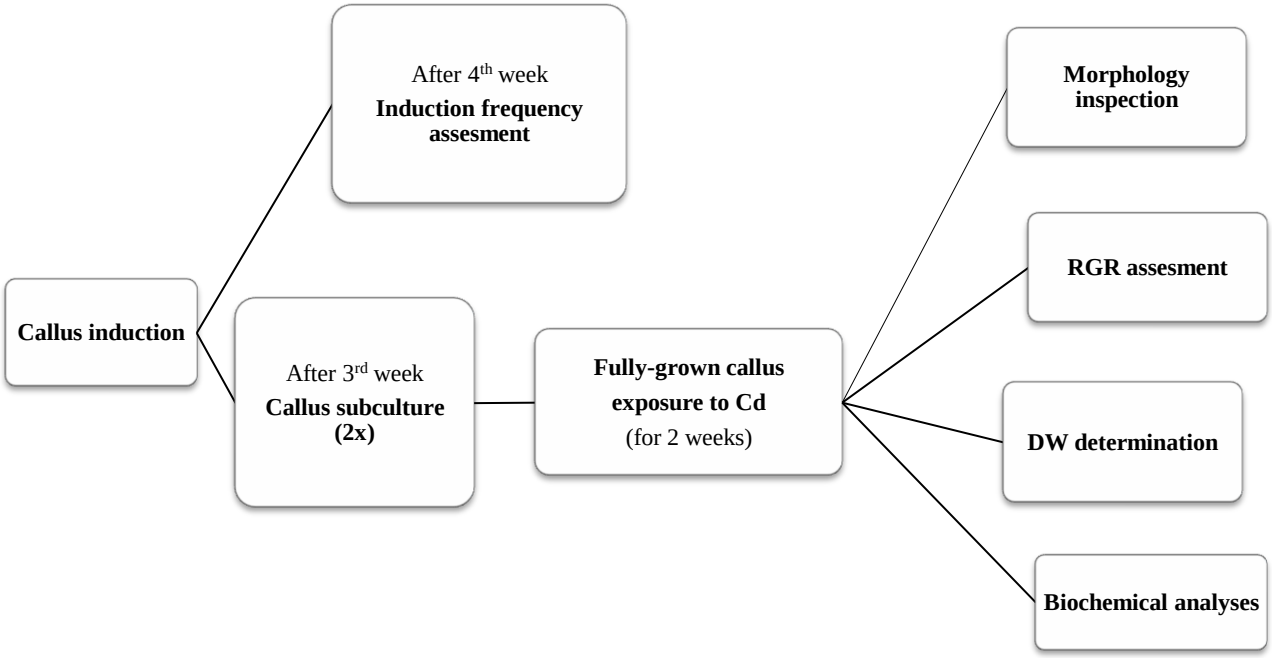
3.3.5.8. Catalase (CAT)

Catalase (EC 1.11.1.6) activity was determined according to the method of Martins et al. (2014), by measuring a decrease in absorbance at 240 nm for 2 min in 50 mM phosphate buffer (pH 7.0) containing 10 mM of hydrogen peroxide. The enzyme activity was defined as the consumption of l μmol H_2O_2 per min and mL.

3.3.5.9. Guaiacol peroxidase (GPX)

Cadmium impacts on guaiacol peroxidise (EC 1.11.1.7) activity was quantified as described by Ranieri et al. (1997). Thereby, it was measured in 1 mL assay mixture consisting of potassium phosphate buffer (0.05 M, pH 6.9), guaiacol (Sigma, St. Louis, USA) as substrate, and enzyme extract. Enzyme extract (callus supernatant) was omitted from the control assay mixture. The absorbance at 470 nm was measured spectrophotometrically after incubation at 23^{o}C for 30 seconds. One unit of enzyme activity was defined as that which brought about a change of 0.01 absorbance unit per minute (Ranieri et al., 1997).

All the experiments described in 3.3.3., 3.3.4. and 3.3.5. were summarised in the following flowchart.



3.3.6. *In vitro* selection of Cd-resistant calli

The appropriate callus type (with reduced Cd sensitivity), determined in the earlier experiments, was used as the starting material for *in vitro* selection. Therefore, the appropriate type of explant (resulting in higher callus frequency) was cultured on the optimised callus induction medium containing either no Cd (even numbered treatments) or 27 µM Cd (54 µM Cd completely inhibited callus formation while 27 µM Cd allowed initiation of some callus and herewith called sub-inhibitory Cd level) (odd numbered treatments). After three weeks of culture, the calli formed were isolated from explants and then grown on the respective callus proliferation medium containing a Cd concentration (109 µM) sub-lethal to callus growth in the different rounds of subculture. Depending on a particular treatment, the number of rounds of callus sub-culture ranged from two to ten but the duration of each round was two weeks (Table 7). To expose both sides of calli to the medium equally, at the end of every week of each subculture round, the calli were turned upside down and placed in contact with the medium (Fig. 25). After each round of sub-culture, non-necrotic (green) patches of calli were excised from the necrotic sections using a sharp scalpel and then placed on fresh medium of the same composition for the next round of sub-culture. Each treatment had four replicates, each containing 50 explants or pieces of calli. All the cultures were kept in the growth room at 21 ± 1°C with continuous lighting.

Table 7. Details of eighteen treatments for selection of Cd-resistant calli.

Treatment number	Cd level* in C.I.[1] medium	Sub-culture rounds[2] on C.P.[3] medium containing sub-lethal Cd level**
1	Sub-inhibitory	2
2	0	2
3	Sub- inhibitory	3
4	0	3
5	Sub- inhibitory	4
6	0	4
7	Sub- inhibitory	5
8	0	5
9	Sub- inhibitory	6
10	0	6
11	Sub- inhibitory	7
12	0	7
13	Sub- inhibitory	8
14	0	8
15	Sub- inhibitory	9
16	0	9
17	Sub- inhibitory	10
18	0	10

[1]: Callus induction (C.I.)
[2]: Each round of sub-culture was two weeks.
[3]: Callus proliferation (C.P.)
*: 27 µM
**: 109 µM

3.3.7. Shoot differentiation

At first, the available protocol for shoot regeneration from Iwa callus (MS+1.7 μM NAA+14.4 μM GA_3 +4.56 μM zeatin) (Wilson et al., 2009) was examined. Afterwards, to optimise a shoot induction protocol, control calli (which were induced and proliferated on Cd-free media) were cultured on half-strength basal MS medium spiked with 2.88, 5.77, 8.66, 11.54 and 14.43 μM GA_3 alone or in combination with different concentrations of BA (2.21, 4.43, 6.65, 8.87 μM) or zeatin (2.28, 4.56, 6.84, 9.12 μM) (Table 12). There were 20 pieces of calli in each of the three replicates of a treatment. The optimised medium was used for shoot regeneration from calli selected in the 18 treatments mentioned in Table 7 (Fig. 27). Shoot regeneration frequency was evaluated for each treatment by recording the number of regenerated shoots in each treatment. All the regenerated shoots were kept on the optimised medium for 4 weeks to develop.

3.3.8. Root induction

To initiate roots from shoots (at least 5 cm long with four developed leaves), the shoots were firstly isolated with a sharp scalpel from their calli and were then cultured on half-strength basal MS medium without any PGR (about 1-2 cm of the base of the excised shoot was inserted into the medium). Root formation and development were assessed after 2 weeks of culture. There were three replicates each containing 20 shoot cuttings.

3.3.9. Plant line establishment and micropropagation

The plantlets regenerated from each treatment (Table 7) were maintained as an individual line (Fig. 28) and was given the number of the treatment. The plantlets were propagated every 4 weeks by culturing shoot tips (nodal segments) in fresh Cd-free half-strength basal MS medium under the same culture conditions as the ones applied to their mother plants. Depending on the length of Cd-resistant callus selection procedure in each treatment, they were subjected to at least three sub-culture rounds (~3 months in total), before they were harvested to undergo *in vitro* screening for Cd resistance (see the work described in Chapter 4).

3.3.10. Statistical analysis

All the experiments were carried out twice and every independent treatment had a minimum of three replicates. Data were subjected to one way analysis of variance (ANOVA, $p \leq 0.05$) following by comparison of the means using Duncan's multiple range test at 5% level of significance. The statistical program SPSS, version 19.0 (Lei et al., 2007) was used.

3.4. Results

3.4.1. Plant materials and culture conditions

In vitro development of the shoot buds (nodal segments) to the whole plantlets occurred after 4 weeks of culture on half-strength basal MS medium under continuous lighting at 21±1°C. The length and diameter of the shoots were about 5 cm and 2 mm, respectively, and each shoot formed 5-8 dark-green leaves (Fig. 9). Formation of axillary shoots was found occasionally. Meanwhile, the root system was well developed.

Figure 9. Fully-grown plantlets developed from nodal segments under *in vitro* conditions.

3.4.2. Sensitivity of plantlets to cadmium stress

Exposure of the excised shoot tips to 218 or 436 μM Cd completely inhibited their further development including formation of roots and new leaves. It also caused severe symptoms of leaf curling and chlorosis (Fig. 10; Table 8). Although 50% reduction in Cd level to 109 μM allowed the excised shoot tips to exhibit their vegetative growth including shoot, root and leaf development, the rates of development were minimum compared to the plantlets grown under lower Cd stress levels (54 and 27 μM Cd). Except necrotic spots on the leaves, no other injury symptom (leaf chlorosis or curling) was seen in 109 μM Cd-treated plantlets. Compared to the control treatment (no addition of Cd to the culture medium), the two lowest Cd treatments (29 and 54 μM Cd) curtailed the vegetative growth about 25 and 35 percent, respectively (Table 8). Minor necrotic spots were observed on the leaf blades of plantlets exposed to 54 μM Cd, whereas injury symptoms were rarely seen in those treated with 29 μM Cd.

Figure 10. Injury symptoms: leaf chlorosis (black arrows) and leaf curling (white arrow) in a plantlet exposed to a lethal Cd concentration (218 μM) for three weeks.

Table 8. The impacts of different concentrations of cadmium added to the culture medium on growth of potato plantlets cv. Iwa. Severity of Cd-induced chlorosis or necrosis was also visually assessed at each Cd exposure level.

Parameters / Cd Con. (µM)	Root length (cm)	Shoot length increase (cm)	Number of new leaves	Chlorosis or necrosis severity
0	5.0±0.60[a]	5.2±0.45[a]	4.8±0.66[a]	not seen
29	3.6±0.71[b]	3.3±0.15[b]	3.6±0.18[b]	not seen
54	3.2±0.02[b]	2.3±0.91[c]	2.3±0.27[c]	minor
109	1.8±0.21[c]	1.0±0.18[d]	1.2±0.47[d]	mild
218	0±0.00[d]	0.2±0.18[e]	0±0.00[e]	major
436	0±0.00[d]	0.1±0.20[e]	0±0.00[e]	severe

- Means with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$).

3.4.3. Callus induction

Callus induction frequency in both types of explants increased in a dose-dependent manner in response to media supplemented with increasing concentrations of picloram (PIC) (Table 9). In response to 12.4 µM PIC, about 83 and 90% of the leaf and internodal explants formed calli, respectively. Supplementation of the medium with 5.37 and 8.05 µM NAA was largely ineffective (below 10%) with respect to callus induction in both types of explants (Table 9). The cytokinin BA at 2.21 µM was found to greatly curtail the callus-inducing effects of picloram (at both 10.35 and 12.42 µM) but slightly elevated callus induction in media with NAA (5.37 and 8.05 µM) for both explant types. Doubling the BA concentration to 4.43 µM and in combination with NAA (at 5.37 µM but not at higher concentrations) resulted in callus induction frequency of about 80 and 66% in the leaf and internodal explants, respectively (Table 9).

Table 9. Effects of different combinations and concentrations of BA, NAA, and PIC on induction of potato callus.

Growth regulators (μM)			Days to callus initiation	Callus induction frequency %		Appearance of callus
BA	NAA	PIC		Leaf	Internode	
0	0	8.28	10-14	31.3±2.9^{c}	39.6±2.0^{c}	Light green, friable
0	0	10.35	10-14	56.6±1.7^{b}	66.0±3.0^{b}	Light green, friable
0	0	12.42	10-14	83.3±1.7^{a}	90.6±2.9^{a}	Light green, friable
0	5.37	0	20-24	8.6±0.6^{e}	4.0±1.0^{fg}	Green, non-friable
0	8.05	0	20-24	3.3±0.5^{f}	1.3±0.3^{g}	Green, non-friable
2.21	0	10.35	14-16	10.3±1.4^{e}	16.6±4.0^{e}	Light green, friable
2.21	0	12.42	14-16	25.3±2.9^{cd}	29.3±5.9^{d}	Light green, friable
2.21	5.37	0	20-24	17.0±1.0^{d}	11.3±1.7^{ef}	Green, non-friable
2.21	8.05	0	20-24	14.6±2.9^{de}	15.3±1.3^{e}	Green, non-friable
2.21	10.74	0	20-24	16.0±2.0^{d}	8.0±1.0^{f}	Green, non-friable
4.43	5.37	0	20-24	80.6±2.4^{a}	66.3±3.1^{b}	Green, non-friable
4.43	8.05	0	20-24	14.6±1.7^{de}	16.6±4.0^{e}	Green, non-friable
4.43	10.74	0	20-24	18.0±1.1^{d}	8.0±1.5^{f}	Green, non-friable

- Data collected after 4 weeks of culture. Means within a column having the same letter are not significantly different by Duncan's multiple range test ($p<0.05$).

The overall external callus morphology seemed to depend on the type of auxin added to the media (Table 9; Fig. 11A&B). In response to picloram, a friable, light-green callus (hereafter referred to as a type-A callus) was formed, while a green, non-friable callus (hereafter referred to as type-B callus) was formed in response to NAA. In addition, as it is depicted in Fig. 11A, type-A callus was initiated all around the explant whereas the other type (type-B) initiation occurred from the (cut) edges of explants (Fig. 11B).

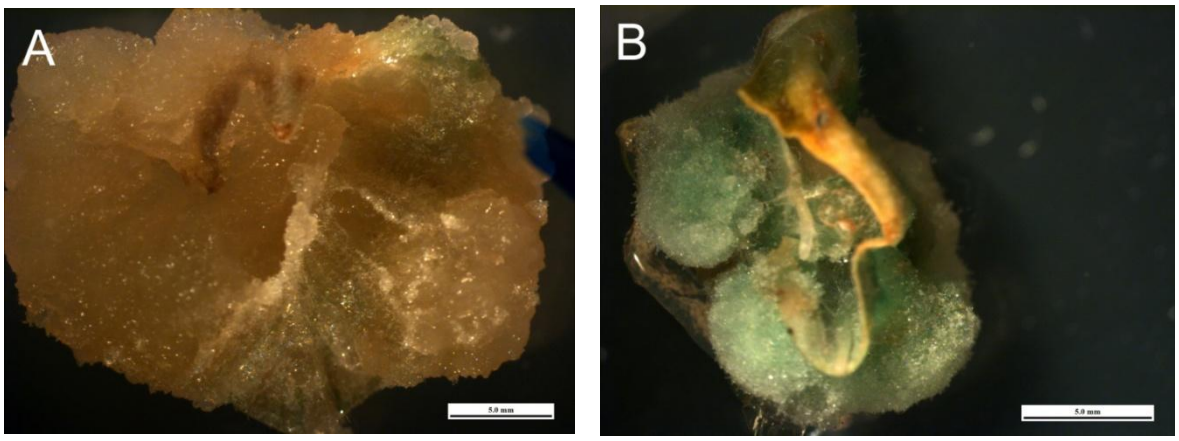


Figure 11. External morphology of the two callus types. (A) Callus type-A with yellow/light green friable cells. (B) Callus type-B with green non-friable cells.

The time to first visible sign of callus formation in the media supplemented with picloram (10-14 days) was faster than in media supplemented with NAA (20-24 days) (Table 9). Subculture of both the callus types on the same media used for their induction was completely successful. Light did not influence the growth of both types of callus as there was no significant difference in callus growth under continuous illumination, 16-h photoperiod or in the dark (Table 10).

Table 10. Effect of different light conditions on proliferation of type-A and -B calli after two weeks of culture.

Growth lighting condition	Type-A callus		Type-B callus	
	Callus proliferation rate (times)	Initial fresh weight of callus (mg)	Callus proliferation rate (times)	Initial fresh weight of callus (mg)
Continuous light	2.4[a]	65.9±7.1	1.3[a]	91.9±4.6
16/8 h photoperiod	2.5[a]	66.1±6.9	1.3[a]	85.0±5.0
Darkness	2.3[a]	70.7±9.0	1.3[a]	83.7±8.4

- Means with the same letter within a column are not significantly different by Duncan's multiple range test ($p<0.05$).

On the other hand, few PGR compositions at particular concentrations were found appropriate for organogenesis rather than callogenesis from the explants. For instance, culture of leaf explants on medium containing 2.21 µM BA + 5.37 µM or higher concentrations of NAA resulted in root formation after 2 weeks of culture (Fig. 12).

Figure 12. Root formation from leaf explants cultured on ½ strength basal MS medium supplemented with 2.21 µM BA and 5.37 µM NAA. The arrows point to the roots that penetrated into the growth medium.

3.4.4. Determination of callus sensitivity to cadmium

3.4.4.1. Cadmium impacts on callus formation

Initiation of both the callus types (A and B) was significantly curtailed (about 30 and 50 percents, respectively) by addition of 27 µM Cd to the respective callus induction media (Figs. 13A&B and 14A&B). Few necrotic tissues were also observed in the leaf explants (14B). On the media supplemented with higher Cd concentrations (54, 109, 218 and 436 µM), neither of callus types was formed from the explants (Table 11 and Figs. 13C&D and 14C&D), and also more necrotic areas were observed in the explants, particularly at 109 µM or higher Cd concentrations (Fig. 14D).

Table 11. Effects of different cadmium concentrations on initiation of type-A and -B calli.

Callus induction medium supplemented with	Type-A callus induction frequency (%)		Type-B callus induction frequency (%)	
	Leaf	Internode	Leaf	Internode
0 µM Cd (control)	82.1±3.3[a]	88.6±2.1[a]	80.5±3.2[a]	69.4±4.6[a]
27 µM Cd	55.9±2.7[b]	63.2±1.1[b]	38.8±5.1[b]	27.7±4.1[b]
54 µM Cd or higher	0[c]	0[c]	0[c]	0[c]

- Mean values with the same letter within a column are not significantly different by Duncan's multiple range test ($p<0.05$).

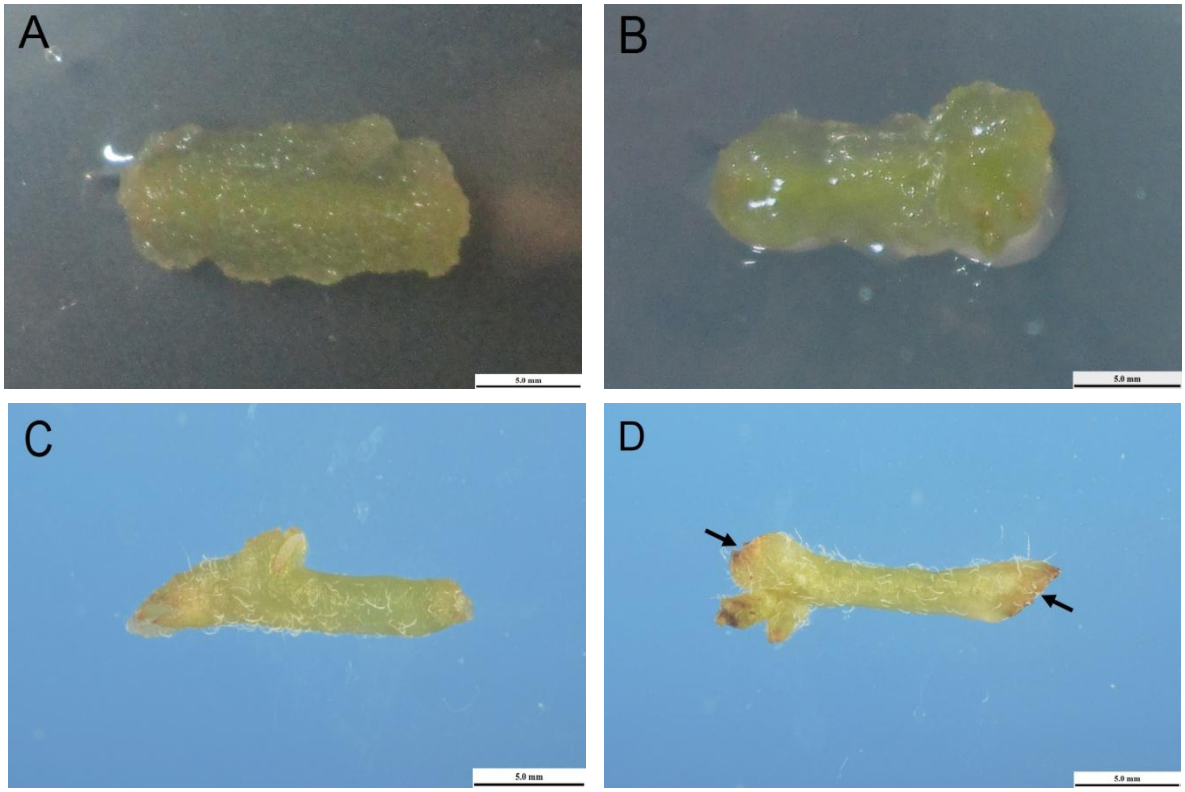


Figure 13. Effects of increasing levels of cadmium on initiation of type-A callus. Internode explants were cultured on the respective callus induction medium containing (A) no Cd (control), (B) 27 μM Cd, (C) 54 μM Cd, and (D) 109 μM Cd for four weeks. Some necrotic tissues (pointed by the arrows) were observed in the explants cultured on the medium supplemented with 109 μM Cd.

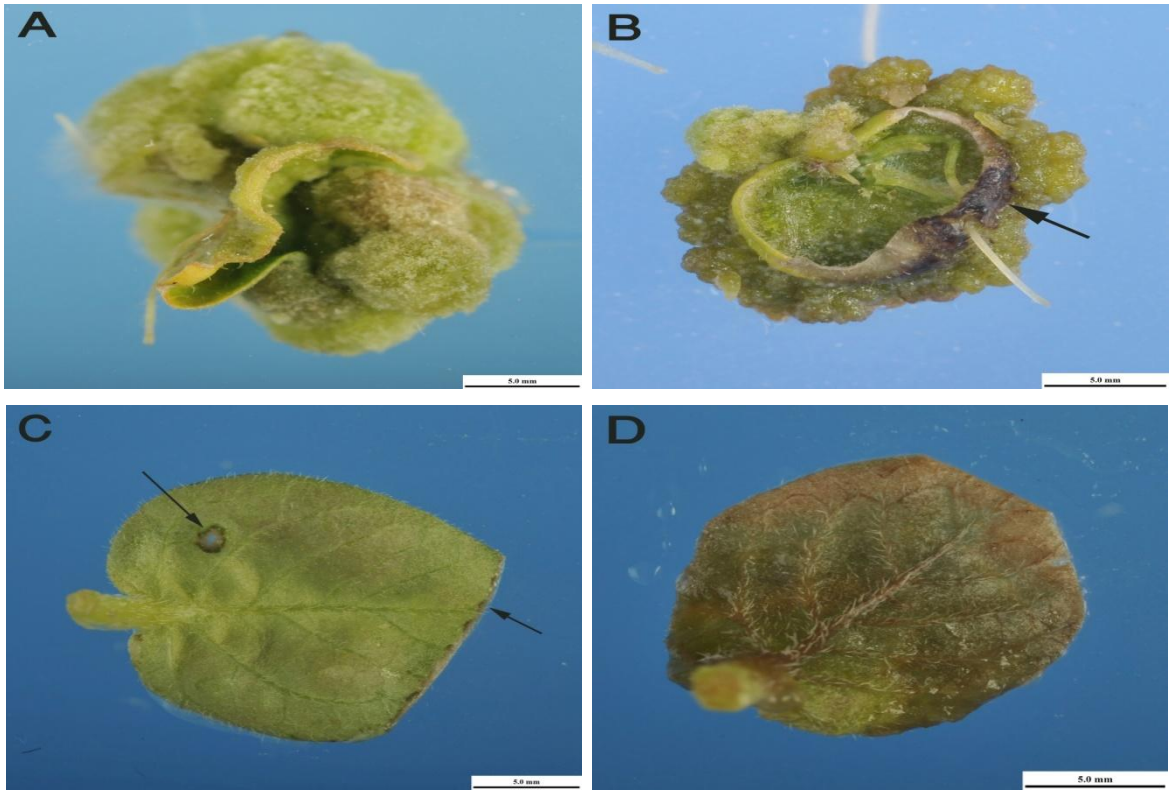


Figure 14. Effects of increasing levels of cadmium on initiation of type-B callus. Leaf explants were cultured on the respective callus induction medium containing (A) no Cd (control), (B) 27 µM Cd, (C) 54 µM Cd, and (D) 109 µM Cd for four weeks. Some necrotic spots (pointed by the arrows) were observed on the explants exposed to 27 or 54 µM Cd, whereas the whole explant colour turn brown on the media spiked with 109 µM Cd.

3.4.4.2. Cadmium impacts on callus morphology

Type-A calli initiated on the medium supplemented with 12.4 µM picloram were subcultured on the same medium with addition of 27, 54, 109, 218 and 436 µM Cd. On the medium containing 27 µM Cd, the colour of some callus patches turned from light green to light brown (Fig. 15), whereas on media supplemented with 54 µM or higher Cd concentrations, the whole callus colour became brown after 14 days of subculture (Fig. 16A&B). In contrast, the morphology of type-B callus initiated on the medium supplemented with 4.43 µM BA and 5.37 µM NAA was not affected by 27 µM Cd and only exhibited a few visible signs of necrosis in response to 54 µM Cd (Fig. 16C&D). Even at 109 µM Cd level, more than half of the callus appeared as non-necrotic (Fig. 17). However, doubling of the Cd concentration to 218 µM, severely affected calli as most of them became necrotic (brown) after two weeks of exposure (Fig. 18A). At the highest Cd level (436 µM Cd), the calli seemed completely necrotic and became shrivelled up (Fig.18B).

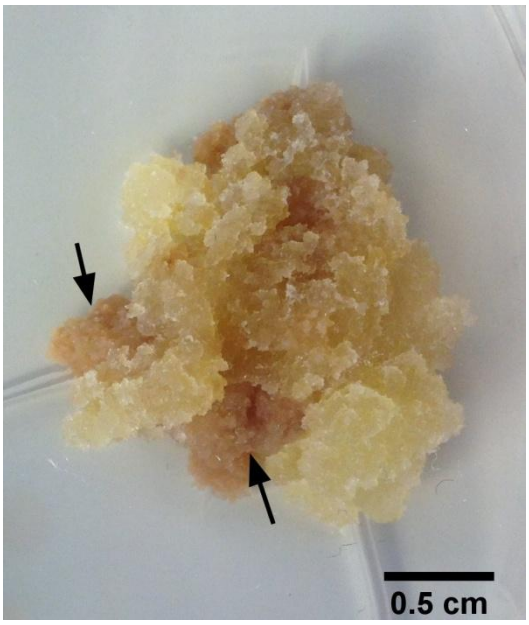


Figure 15. The typical external morphology of type-A callus after two weeks of culture in the presence of 27 µM Cd. The regions of the callus that turned light brown are indicated by the arrows.

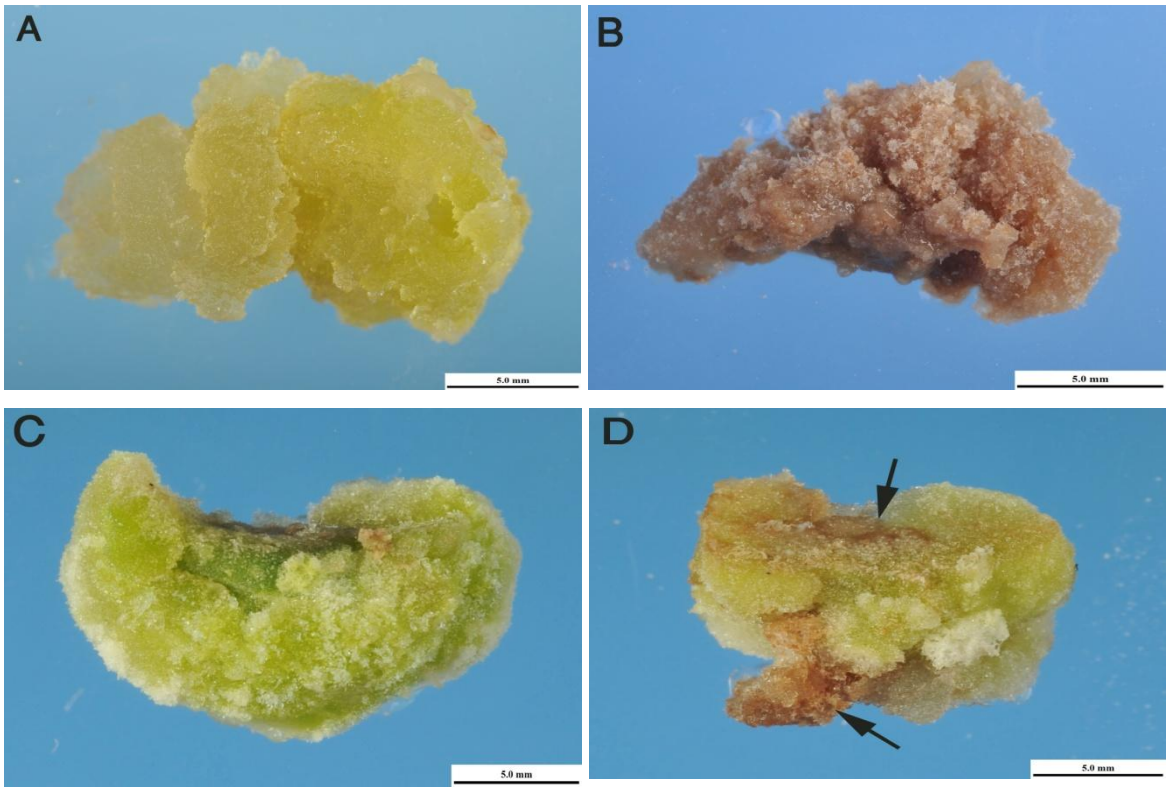


Figure 16. (A) Light green friable type-A callus (control), (B) Type-A callus treated with 54 µM cadmium for 14 days, (C) Green compact, non-friable type-B callus (control) and (D) Type-B callus treated with 54 µM cadmium for 14 days. The arrows point to necrotic tissues.

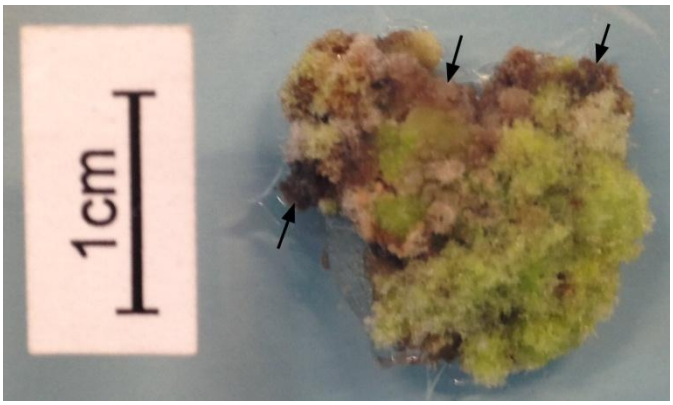


Figure 17. Impacts of 109 µM Cd on the external morphology of a growing type-B callus. The arrows point to necrotic tissues.

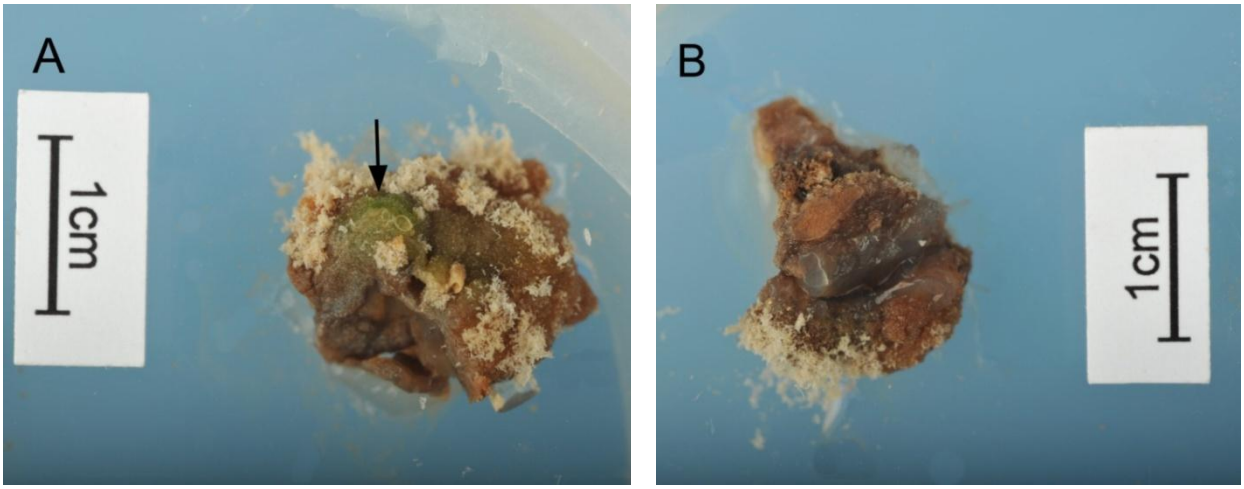


Figure 18. Type-B callus treated with (A) 218 µM Cd and (B) 436 µM Cd for 14 days. The arrow points to non-necrotic tissue.

3.4.4.3. Cadmium impacts on relative growth rate of callus

On medium without addition of Cd, type-A callus exhibited 20 percent higher relative growth rate (RGR) than type-B callus (Fig. 19). In response to 27 µM Cd in the culture medium, type-A callus exhibited significant decrease (30%) in RGR compared to the control (Fig. 19). There was a further substantial decrease (~45%) in RGR of type-A callus when the Cd concentration was increased to 54 µM. Callus growth was completely inhibited at 109 µM or higher Cd concentrations. In contrast, there was no significant difference between RGRs of type-B callus in response to 0 and 27 µM Cd spiked with the medium. On the medium supplemented with 54 µM Cd, RGR of type-B callus was about 35%, which was seven times higher than that of type-A callus (6%) at the same exposure level. Even in response to 109 µM Cd, type-B calli still managed RGR of just above 20% (Fig. 19).

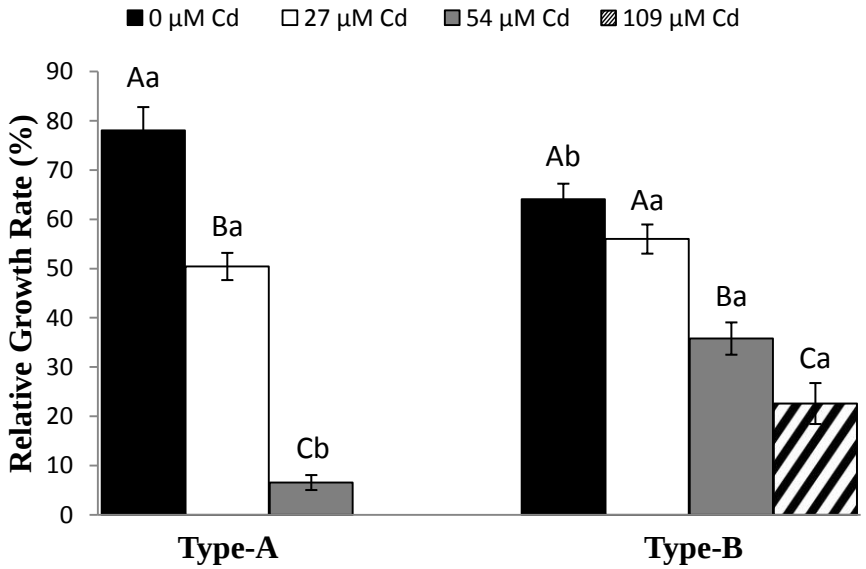


Figure 19. Effects of different concentrations of cadmium chloride on the relative growth rates of the two callus types (A and B). No growth was seen in type-A callus treated with 109 µM Cd. The vertical bars show means ± standard errors (n=3). Mean values with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$). Upper and lower case letters are used for comparison within a same callus type and between the callus types respectively.

3.4.4.4. Determination of dry weight

Comparison of the dry weights of the two callus types grown on the control media (0 Cd) showed that type-A callus basically had a higher water content than type-B callus (Fig. 20). With addition of 27 µM Cd to the growth medium, the dry weight of type-A callus was reduced by 10 percent, whereas that of type-B callus was not reduced significantly. Callus treatment with 54 µM Cd caused water loss in both the callus types. However, this was much noticeable in type-A callus (45% reduction compared to the control callus DW) than type-B (17%) (Fig. 20). Further increase in the Cd level of media (to 109 µM Cd) significantly affected the dry weight of type-B callus (a further 10% reduction) while it had no significant impact on the dry weight of type-A callus.

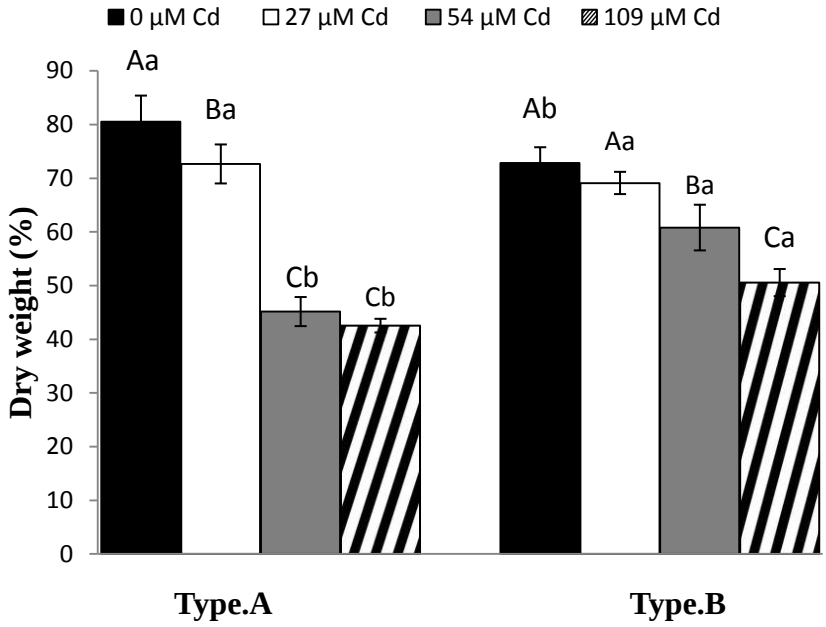


Figure 20. Effects of different concentrations of cadmium chloride on the dry weights of the two callus types (A and B). The vertical bars show means ± standard errors (n=3). Mean values with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$). Upper and lower case letters are used for comparison within a same callus type and between the callus types respectively.

3.4.4.5. Hydrogen peroxide

Under control condition (no Cd), hydrogen peroxide (H_2O_2) level in the type-B callus was found to be relatively higher (with 30 mmol/g FW difference) than that in type-A callus (Fig. 21). Although both callus types (A and B) showed significant increases in H_2O_2 levels in response to the 27 µM Cd treatment compared to the respective controls (calli grown on media without addition of Cd), the increase in callus type-A was about double of that in type-B callus (Fig. 21). The H_2O_2 levels after exposure to 54 µM Cd were elevated further in both callus types. Treatment with 109 µM Cd in comparison with the other Cd concentrations brought about the greatest increase in H_2O_2 levels in type-B callus, but there was no further change in type-A callus (Fig. 21).

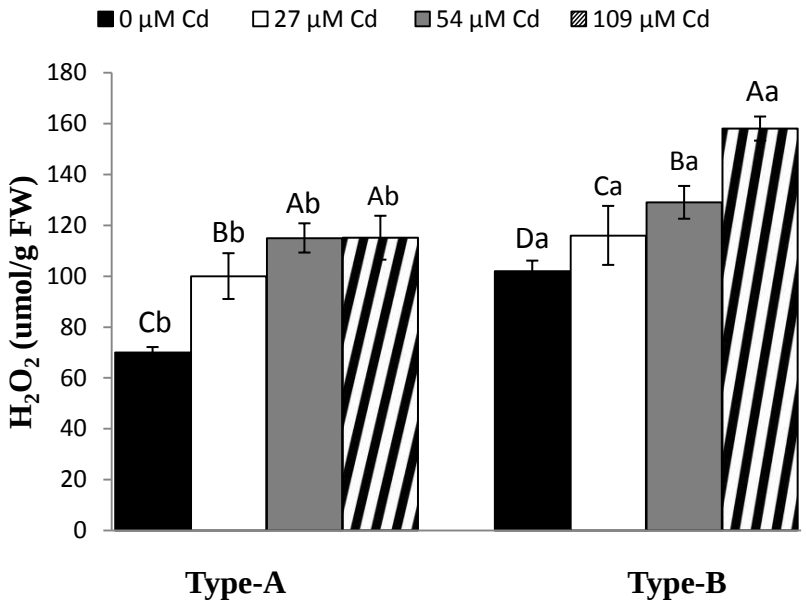


Figure 21. Effects of different concentrations of cadmium chloride on hydrogen peroxide level in the two callus types (A and B). The vertical bars show means ± standard errors (n=3). Mean values with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$). Upper and lower case letters are used for comparison within a same callus type and between the callus types respectively.

3.4.4.6. Antioxidant activities

In the absence of cadmium, type-B callus exhibited higher superoxide dismutase (SOD) and catalase (CAT) activities than type-A callus (Figs. 22&23), while there was no difference in guaiacol peroxidase (GPX) activity between the two types of callus (Fig. 24). Addition of 27 µM cadmium to the culture medium increased SOD and CAT activities in both types of callus. In contrast, GPX activity was decreased in type-A callus while there was no change in type-B callus. At higher concentrations of cadmium added to the medium, SOD, CAT and GPX activities were decreased in type-A callus, but type-B callus was less affected.

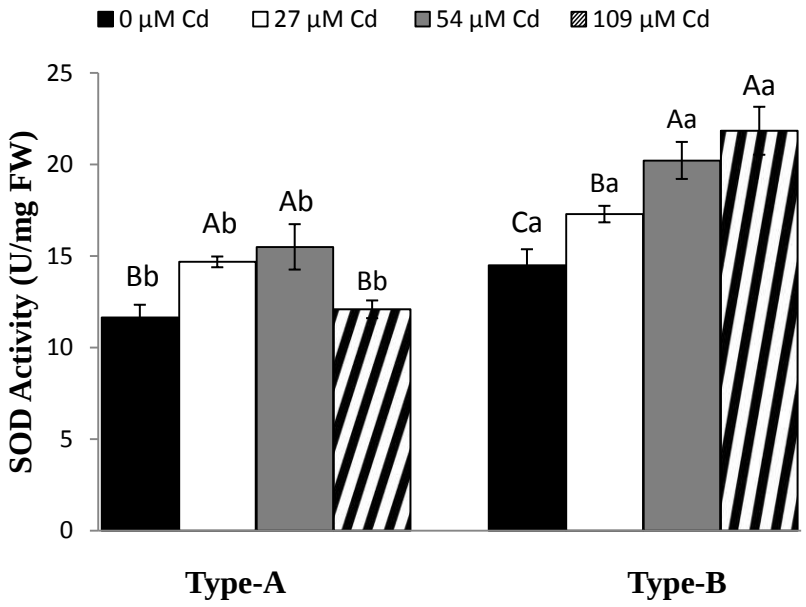


Figure 22. Changes in superoxide dismutase (SOD) activity in the two callus types (A and B) treated with 0, 27, 54 and 109 µM cadmium chloride for 14 days. The vertical bars show standard errors (n=3). Mean values with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$). Upper and lower case letters are used for comparison within a same callus type and between the callus types respectively.

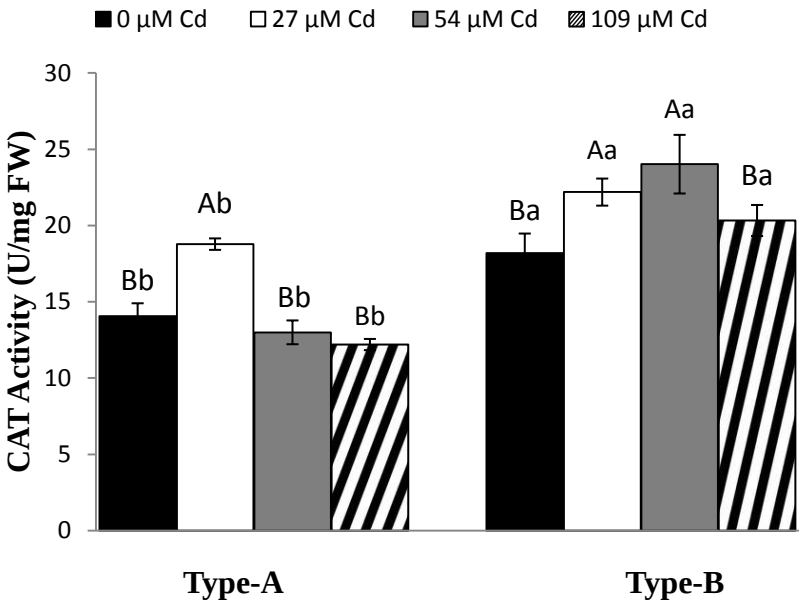


Figure 23. Changes in catalase (CAT) activity in the two callus types (A and B) treated with 0, 27, 54 and 109 µM cadmium chloride for 14 days. The vertical bars show standard errors (n=3). Mean values with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$). Upper and lower case letters are used for comparison within a same callus type and between the callus types respectively.

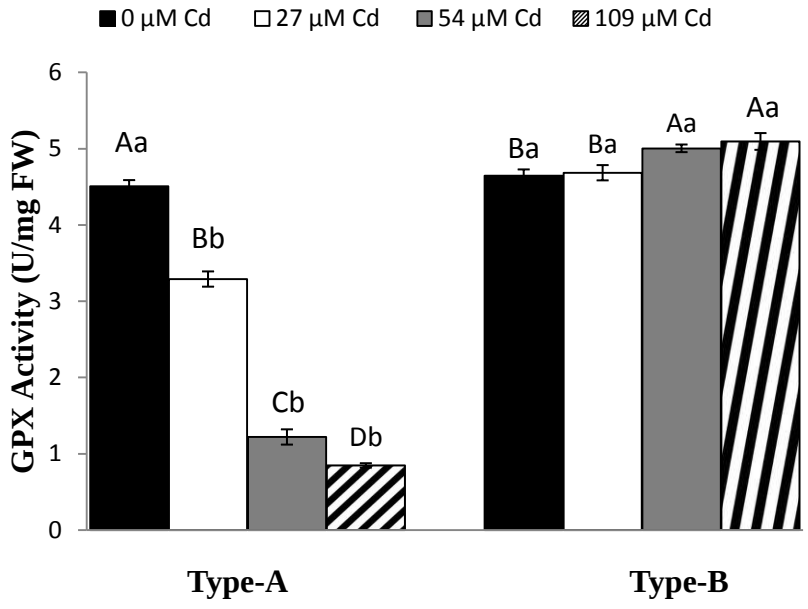


Figure 24. Changes in guaiacol peroxidase (GPX) activity in the two callus types (A and B) treated with 0, 27, 54, and 109 µM cadmium chloride for 14 days. The vertical bars show standard errors (n=3). Mean values with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$). Upper and lower case letters are used for comparison within a same callus type and between the callus types respectively.

3.4.5. *In vitro* selection of Cd-resistant calli

Eighteen treatments differed in terms of absence or presence of Cd supplementation (at a sub-inhibitory level: 27 µM) in the callus initiation medium, or/and number of subculture on the callus proliferation medium spiked with a higher but still sub-lethal Cd concentration (109 µM) (Table 7). Study of external morphology of callus showed that occurrence of necrosis in callus was not correlated with direct contact of callus with the surface of HM-containing medium (Fig. 25 A&B). As it can be seen (Fig. 25B), the side of the callus that was in contact with the medium within the first week before being turned upside down, was not necessarily necrotic. Due to the different durations of treatments (ranging from one to five months), selected calli from the final rounds of treatments were transferred to the optimised shoot regeneration medium at different times.

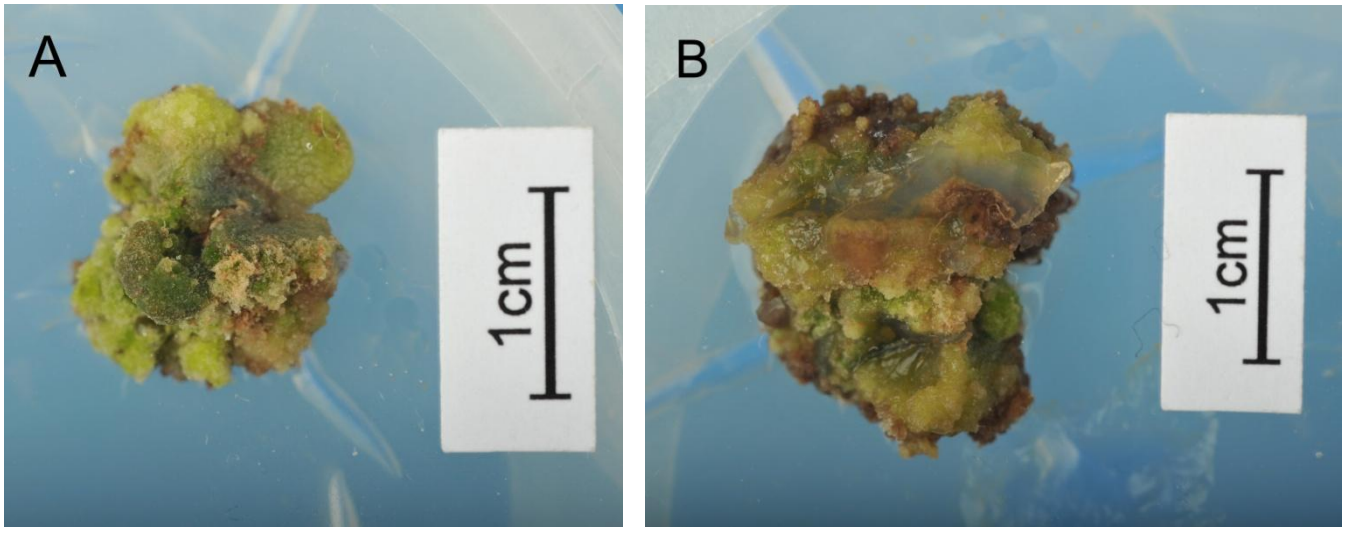


Figure 25. Appearance of a piece of callus before (A) the first and (B) second week of subculture on callus proliferation medium containing 109 µM Cd (round 1, treatment 4).

3.4.6. Plant regeneration

Shoot regeneration according to a previous protocol (Wilson et al., 2009) was completely unsuccessful. In contrast, callus culture on three of the fourteen PGR treatments here resulted in more than 50 percent shoot induction (Table 12). Thus, half-strength basal MS medium containing 11.54 μM GA_3 with 8.87 μM BA or 9.12 μM zeatin was found to be the optimised shoot initiation medium. More than 75% of control type-B calli cultured on either of these two media formed shoots (Table 12). The first sign of shoot induction was observed a few days (1 to 3 days) earlier in the calli cultured on the medium supplemented with BA than zeatin. Therefore, due to this preference for BA in the medium and a higher shoot regeneration rate, *in vitro* Cd-selected calli were cultured on half-strength basal MS medium containing 11.54 μM GA_3 + 8.87 μM BA. Neither 11.54, nor 14.43 μM GA_3 alone could trigger callus cells to form any shoot buds (Table 12). A combination of 14.43 μM GA_3 and different concentrations (4.56, 6.84 and 9.12 μM) of zeatin mainly resulted in root formation rather than shoot formation. All the same 14 media to regenerate shoots from type-A callus was not successful. For shoot buds to develop into shoots with leaves, they were kept on the shoot regeneration medium for 3 to 4 more weeks (Fig. 26). Root formation from control shoots (regenerated from calli that were not exposed to Cd) occurred in PGR-free half-strength MS medium. Consequently, this medium was chosen for root regeneration.

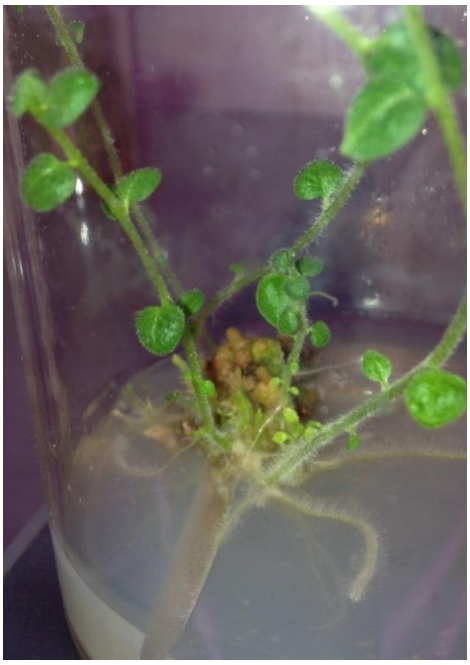


Figure 26. Three-week-old shoots with leaves.

Table 12. Effects of different combinations and concentrations of GA_3, BA and zeatin on shoot regeneration from type-B callus (control).

Growth regulators (μM)			Days to shoot initiation	Shoot regeneration frequency %
BA	Zeatin	GA_3		
0	0	11.54	-	0^{f}
0	0	14.43	-	0^{f}
4.43	0	11.54	26-30	23.3 ± 1.1^{c}
6.65	0	11.54	26-30	26.6 ± 0.7^{c}
8.87	0	11.54	26-30	83.1 ± 1.2^{a}
0	4.56	11.54	28-33	17.3 ± 1.8^{d}
0	6.84	11.54	27-33	16.3 ± 1.9^{d}
0	9.12	11.54	27-33	77.4 ± 1.1^{ab}
4.43	0	14.43	26-30	56.2 ± 1.4^{b}
6.65	0	14.43	26-30	21.0 ± 2.2^{cd}
8.87	0	14.43	26-30	11.6 ± 2.0^{de}
0	4.56	14.43	29-32	9.6 ± 1.5^{e}
0	6.84	14.43	29-32	9.0 ± 1.1^{e}
0	9.12	14.43	27-31	9.7 ± 0.4^{e}

- Data collected after 5 weeks of culture. Means within a column having the same letter are not significantly different by Duncan's multiple range test ($p<0.05$).

The results showed that the frequency of shoot regeneration from *in vitro* selected calli was correlated with the duration of Cd exposure (within induction and/or proliferation stages) in which they were selected (Table 13). Prolonged Cd exposure curtailed shoot regeneration frequency up to about 30 percent from calli exposed to Cd for more than 5 months. While, the shoot induction frequency from calli selected through short-term Cd exposure including treatments 1, 2, 3, 4 and 6, was not statistically different to that from control calli (experienced no Cd) (83 percent) (Table 13).

Table 13. Shoot regeneration frequency from selected calli in the 18 treatments.

Treatment number	Shoot induction frequency
1	84.6±1.4[a]
2	82.1±1.0[a]
3	81.4±1.2[a]
4	82.2±0.9[a]
5	80.13±0.9[a]
6	78.8±1.3[ab]
7	75.7±2.1[b]
8	78.3±1.9[ab]
9	73.0±1.2[b]
10	70.4±1.7[bc]
11	70.3±1.7[bc]
12	68.2±2.2[c]
13	74.6±1.3[b]
14	65.7±1.5[c]
15	62.9±2.0[cd]
16	60.4±2.1[d]
17	60.5±1.1[d]
18	53.1±1.0[e]

- Based on the duration of Cd exposure, the treatments were arranged in descending order.

A few shoots of the regenerated plantlets were selected randomly to examine shoot morphology using a stereo microscope (OLYMPUS-C011). It was found that all the shoots originated from calli exposed to Cd only at the proliferation stage (the even-numbered treatments in Table 13) did not form lateral shoots

(Fig. 27 A&B), whereas most of the shoots regenerated from calli exposed to Cd during both the induction and proliferation stages (odd-numbered treatments in Table 13) exhibited branching (Fig. 27 C&D).

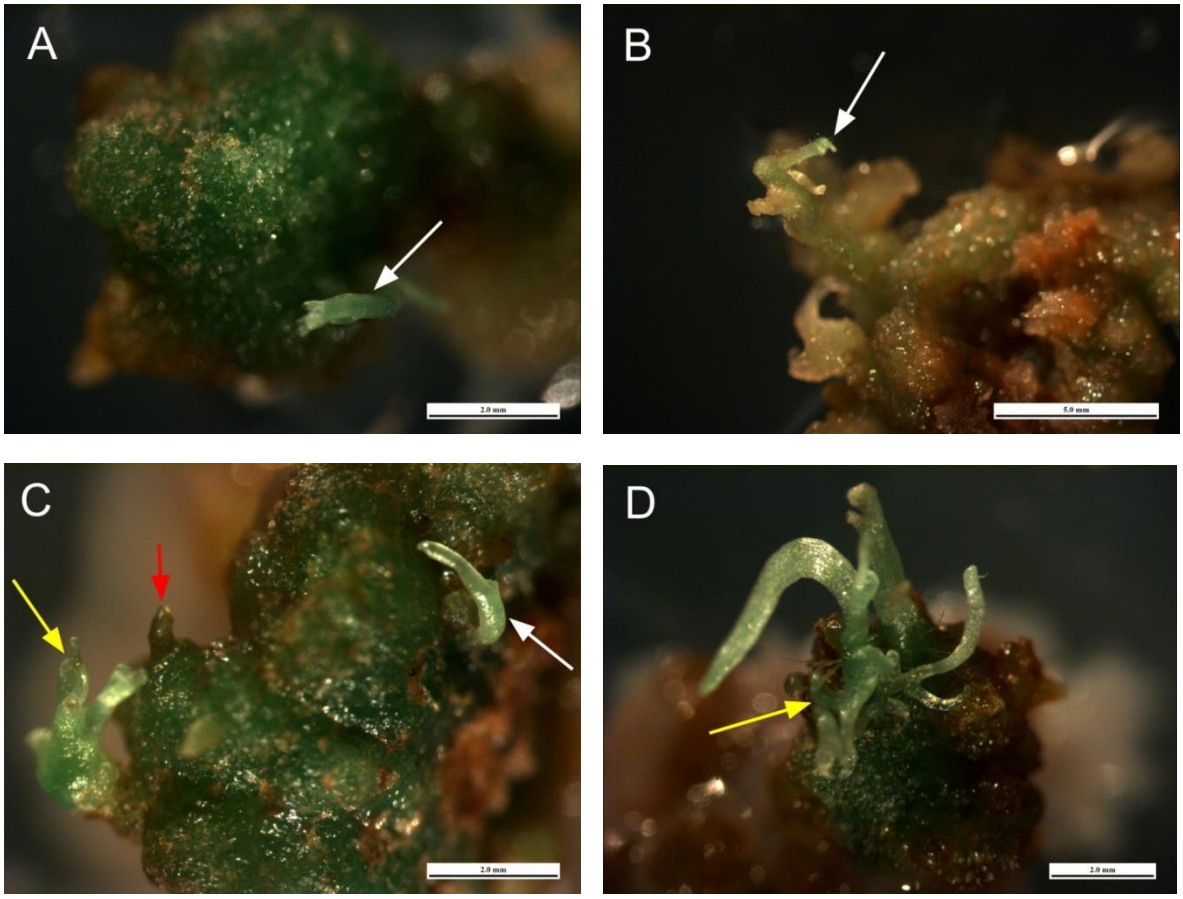


Figure 27. Effects of Cd exposure timing on shoot branching. (A&B) Un-branched shoot buds (white arrows) originated from calli experienced Cd only during proliferation; (C&D) Branched shoot buds (yellow arrows) originated from calli experienced Cd from earlier (initiation stage). The red arrow points to a new shoot bud.

3.4.7. Line establishment

The plantlets obtained in each of the 18 treatments (Table 13) were regarded herewith as an individual line of Iwa cultivar (18 lines in total). They were named from 1 to 18 corresponding to the treatments of the calli (1 to 18). The plantlets of two individual lines, 4 and 7, grown under *in vitro* conditions were shown as the examples of the plantlets obtained (Fig. 28A&B). Micropropagation in half-strength basal MS medium was found to be useful for maintenance of the plantlets of all the lines.

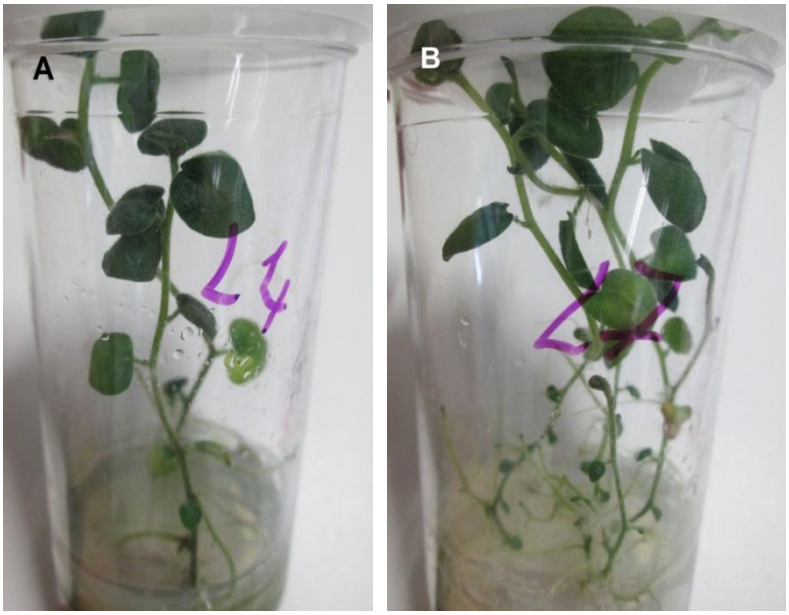


Figure 28. Examples of well-grown plantlets regenerated from an (A) even-numbered and (B) odd-numbered lines (see Table 13).

3.5. Discussion

HM resistance promoted through the somaclonal variation can vary greatly in terms of the underlying mechanisms ranging from tolerance to exclusion (Mohan Jain, 2001; Viehweger, 2014). This is mainly due to the random nature of somaclonal variation. This has given rise to some criticisms on the *in vitro* selection approach. In the case of Cd resistance, to date, *in vitro* breeding has been only exploited for the purpose of enhancing Cd tolerance in hyper-accumulator species including *Brassica juncea* L. (Nehnevajova et al., 2007; Shekhawat et al., 2010), and has not been employed for obtaining resistance through the other possible mechanism (reduced Cd uptake) in any crop species. In this research, for the first time, somaclonal variation was used to improve Cd resistance of potato, a food plant, which is one of the most important staple crops globally. Obviously, somaclones with lower Cd uptake and/or accumulation would be of interest.

The Cd concentration (218 μM) found to be lethal to Iwa plantlets was also lethal for potato cv. Premiere1 (208 μM Cd) by Domazlicka and Opatrny (1989). However, this cultivar was relatively more sensitive than the Iwa cultivar to 20 μM Cd. At this Cd level, root elongation in cv. Premiere1 was decreased by up to 50 percent of that in control plantlets, whereas Iwa plantlets were less affected. At this level (20 μM Cd), chlorosis and severe leaf curling were also observed in Premiere 1 plantlets (Domazlicka and Opatrny, 1989), while none of these symptoms was seen in Iwa plantlets.

As somaclonal variation more likely occurs in callus cells (Bajaj, 1990), induction of callus with the desirable trait is a key to successful *in vitro* selection programmes. It is well-proved that different types of plant growth regulators may be able to induce different morphologically dissimilar calli from the same or different explants of the same plants (Akaneme and Ene-Obong,

2008; JayaSree et al., 2001; Perez-Clemente et al., 2013; Visarada et al., 2002). For instance, JayaSree et al. (2001) induced two distinct types of calli from potato leaf explants by using two different auxins: NAA and 2,4-D but same cytokinin source (BA). The culture on NAA-containing media resulted in initiation of a compact type of callus which was found suitable for organogenesis, whereas the culture on the media supplemented with 2,4-D caused induction of nodular embryogenic callus which was capable for organogenesis as well (JayaSree et al., 2001).

Similarly here, application of NAA and other auxin type: PIC resulted in initiation of two morphologically distinguishable calli. Picloram alone could elicit formation of a type of soft/friable callus (type-A) while BA in combination with NAA resulted in the formation of a more compact or non-friable type-B callus. This can be related to the influential roles of auxins on determination of callus texture. It seems that NAA can trigger cells in the explant cell to form compact callus, whereas, both PIC and 2,4-D led to initiation of a friable type of callus on the explants. This may be attributed to the similarities of these two PGRs in terms of having chlorine in their chemical structures. On the other hand, culture of neither leaf, nor internodal explant on callus induction medium established by Wilson et al. (2009) resulted in callus formation.

The picloram-induced type-A callus was faster growing than type-B callus. Unlike the friable callus obtained previously (JayaSree et al., 2001), the friable callus (type-A) initiated here, did not respond to organogenesis inducing treatments. This callus type would be most useful as starting material for initiation of a cell suspension culture owing to the relative ease of its fragmentation in a liquid medium. On the other hand, the compact type (type-B) was found really capable for organogenesis. However, both leaf and internodal

explants of potato exhibited a high frequency of callus formation in response to picloram alone or BA in combination with NAA.

Based on the primary aim of the present study which was to determine which callus type would be more preferable for initiating a programme to select somaclonal variants with enhanced resistance to Cd, their morphological and biochemical characters were compared after exposing to the different levels of Cd stress. Thereby, traits of interest which were low Cd sensitivity and high initiation and organogenesis potential were identified in type-B callus. This was the first time that the implications of callus type for selection of the potential somaclonal variants with improved Cd resistance have been assessed.

Comparative studies of friable and non-friable calli in tobacco and sugarcane have found differences in the chemical composition of cell walls between friable and non-friable callus types (Halmer and Thorpe, 1976; Liners et al., 1994). Here we found that the non-friable potato callus initiated on medium supplemented with BA and NAA was more tolerant to 27-109 µM Cd than the friable callus induced on medium supplemented with picloram. This was correlated with higher antioxidative enzyme activities in non-friable calli than in friable calli. It has been shown that an important detoxification mechanism in plants involves immobilisation of toxic metals in the cell wall (Phang et al., 2011). It remains to be determined if altered cell wall composition in the friable potato callus might be correlated with its relatively reduced resistance to Cd than the non-friable potato callus.

Cadmium toxicity in plant cells is mainly related to elevated levels of oxidative stress (Schutzendubel and Polle, 2002). On the other hand, resistance to this toxicity might be linked to enhanced activities of antioxidative enzymes (Chen et al., 2008). As superoxide dismutase (SOD) is a key enzyme in catalysing the dismutation of stress-induced free radicals (e.g. O_2^-) into hydrogen peroxide

(H_2O_2), assessment of its activity could uncover the respective antioxidative mechanisms employed by the two callus types. In response to an increase in Cd concentration up to 54 µM Cd, H_2O_2 content was found to increase in both types of calli and was in agreement with the increased SOD activity. In *Brassica juncea* callus, SOD activity was also found to be Cd concentration-dependent, up to 50 µM (Shekhawat et al., 2010). However, SOD activity in type-A callus was found to be more sensitive to the higher Cd concentration (109 µM) whereas SOD activity was increased further in type-B callus. Moreover, H_2O_2 level in the control and type-B calli exposed to Cd were higher than type-A calli but type-B calli were less affected by Cd. This suggests that type-B calli were better protected from Cd-induced reactive oxygen species (ROS) stress which might be counteracted by antioxidative defence activities such as SOD activity etc. Xu et al. (2011) also found a pivotal role for SOD in tolerating Cu-induced oxidative stress in *philoxeroides* callus. Assessing the activities of peroxidases and catalase which are the two main enzymes converting H_2O_2 (generated by SOD action) into harmless water (H_2O), revealed more differences between antioxidative capacities of the callus types. In response to increase in Cd concentrations, the ascending levels of GPX and CAT activities in type-B callus suggest that the less Cd injury symptoms observed in type-B callus could be associated with its enhanced antioxidative capacity therein. In contrast, it seems that H_2O_2 in type-A callus was not scavenged efficiently by neither GPX at all Cd levels, nor CAT at high levels (54 and higher µM Cd). The contiguous functions of GPX and CAT led to a drop in H_2O_2 level in *Alternanthera philoxeroides* callus under Cd stress (Xu et al., 2011). Due to the toxicity of H_2O_2 in the plant cells (Winterbourn, 1995), a lack of efficient H_2O_2 depletion system can be a reason for the observed higher sensitivity to the stress factor (Cd).

Even though gluthatione-ascorbate cycle enzymes including ascorbate peroxidise (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) were not assessed in the present study, they might not be influential in the callus as this cycle is principally active in the chloropolasts (Fornazier et al., 2002).

A conclusion from the comparative study of the two types of potato callus was that starting from the non-friable callus (type-B) would be the preferred strategy for selection of somaclonal variants with enhanced Cd resistance. Therefore, based on the lower Cd-sensitivity and higher shoot regeneration potential of type-B callus, it was chosen for the further experiments on *in vitro* selection for promising Cd-resistant somaclonal potato variants.

Table 14. *In vitro* plant cell selection studies carried out aiming to enhance HM resistance in different plant species.

Plant species	Initial plant material	Heavy metal/ Concentration	Exposure time	Reference
Setaria italic (foxtail millet)	Callus	240 µM Zn	4 weeks	(Samantaray et al., 1999)
Echinochloa colona (jungle rice)	Callus	5 mM Cr + 7.6 mM Ni	4 weeks	(Samantaray et al., 2001)
Brassica spp.	Callus	240 µM Zn or 800 µM Mn	4 weeks	(Rout et al., 1999)
Setaria italic (foxtail millet)	Callus	5.7 mM Ni	4 weeks	(Rout et al., 1998)
Cucumis sativus (cucumber)	Cell line	100 µM Cd	6 weeks	(Gzyl and Gwóźdź, 2005)
Solanum lycopersicum (tomato)	Cell line	≤6 mM Cd	6 months	(Goldsbrough, 1991)
Populus nigra (black poplar)	Callus	150, 250 µM Cd	3 weeks	(Iori et al., 2012)
Brassica juncea (Indian mustard)	Callus	10, 20 µM Cd or Pb	6 months	(Nehnevajova et al., 2007)
Nicotiana tabacum (tobacco)	Callus	60 µM Cu	4 weeks	(Rout and Sahoo, 2007)
Nicotiana tabacum (tobacco)	Callus	2, 5 mM Mn	8 months	(Santandrea et al., 1998)
Oryza sativa (rice)	Callus	250-1000 µM Al	20 weeks	(Van Sint Jan et al., 1997)
Oryza sativa (rice)	Callus	87, 175 µM Al	4 weeks	(Roy and Mandal, 2005)

In the previous *in vitro* breeding studies summarised in Table 14, the implication of HM exposure duration on the success of a selection programme was not investigated as all calli were subjected to a specific treatment regime (same exposure period and timing). Moreover, selection has been mainly performed within callus proliferation stage and no attempt has been done to expose callus cells to HM earlier (from callus initiation stage), which may be very influential. Similarly, for improving potato (cv. Iwa) resistance to common scab disease, potato callus was firstly initiated from stem internodes on a solidified medium, and then callus cells were exposed to thaxtomin-A in liquid medium (Wilson et al., 2009). It is clear that HM exposure duration should be long enough to give a chance to callus cells to generate mutations of interest (HM resistance), but "how long is enough" is unknown. Hence, in the present study, based on the two variables: early Cd exposure (started from callus induction stage) and exposure duration (varied from 1 to 5 months), 18 different treatments were designed (Table 7). Morphological and biochemical studies showed that 54 µM Cd is the Cd concentration inhibitory to initiation of both types of calli (A and B) and therefore 27 µM Cd was used as the sub-inhibitory Cd concentration in the *in vitro* selection treatments (Table 7). On the other hand, largely different Cd levels (a 4-fold difference) inhibited growth of type-A and type-B calli as proliferation of type-A and type-B calli was completely prevented on 54 and 218 µM Cd-containing medium, respectively. This concentration (218 µM Cd) was also found to be lethal to the mother plantlets as well.

An implication of the results obtained is that presence of a cytokinin source at a certain concentration in culture medium has a greater impact on shoot regeneration than cytokinin type. Furthermore, the results confirmed the influence of Cd exposure period on shoot regeneration potential. It would be due to loss of callus cell totipotency by DNA methylation or physical changes

in chromosomes (Atwell et al., 1999). Hence, prolonged HM exposure would make regeneration stage of *in vitro* selection difficult while it may not be necessary.

On the other hand, the other variable (exposure timing) was interestingly found to be correlated with shoot branching. Basically, axillary bud growth to develop a branch is under control of specific genes such as *BRC1* identified in *Arabidopsis*, but environmental stimuli can also affect this through hormonal signals (Aguilar-Martínez et al., 2007). Cadmium stress was found as one of the inhibitors of axillary bud development (Lefevre et al., 2005). Therefore, the results presented in 3.4.6., may be related to neutralizing impact of early Cd introduction (from callus formation stage) on the arrest of axillary bud development. To the best of my knowledge, it was the first time that relationship between shoot branching and timing of selecting agent application has been observed through an *in vitro* selection programme. These parameters can be very critical in determining success of an *in vitro* selection programme (improvement) or failure (no improvement or even undesirable changes).

To avoid recovery of plant regenerants with epigenetic changes, exposure to a sub-lethal Cd concentration was preferred to stepwise Cd exposure method which is a prolonged procedure. Many authors believed that the problem of epigenetic adaptation of non-tolerant cells can be addressed by short-term or one-step selection (Rai et al., 2011). Altered DNA methylation has been known as one of its reasons. However, all the plantlets regenerated from selected calli were subjected to culture for a minimum of three months under stress-free condition (Cd-free) to ensure avoiding transient variants. To sum up, in the design of the present study, optimum methods recommended by previous *in vitro* breeding studies were used as well as investigations into roles of other *in vitro* selection parameters that had been neglected previously.

Chapter 4- *In vitro* Screening of Potato Plant Lines Regenerated from Cd-treated Calli for Cadmium Resistance

4.1. Introduction

In vitro screening is an assessment method to identify the most promising variants of interest (specifically those with enhanced Cd resistance here) among the regenerants after selection for the trait of interest. Indeed, the *in vitro* culture-based technique is a promising alternative to costly labour-intensive field trials which can be problematic due to spatial heterogeneity of soil chemical and physical properties, and seasonal fluctuations (Albiski et al., 2012; Zhang and Donnelly, 1997). Furthermore, as *in vitro* screening is conducted under aseptic conditions, the results obtained would not be complicated by potential interferences/interactions with microorganisms in comparison with field trials.

Application of *in vitro* tissue culture technique is not limited to generation and selection of resistant plant materials (e.g. callus) to abiotic stresses, but it can also be subsequently employed for screening of desirable plants after plant regeneration from selected calli. Like *in vitro* selection involving calli, *in vitro* screening of putative somaclonal variants is conducted under controlled laboratory conditions so that it is independent of climatic conditions making its efficiency much higher than *in vivo* screening methods including field trials. Moreover, application of *in vitro* screening has enabled plant breeders to evaluate numerous genotypes or lines rapidly at the same time (large-scale screening).

Screening of the different plants can be started either immediately after regeneration from selected calli surviving selection against the HM under investigation or after an interval in which the regenerants are sub-cultured under HM-free conditions (Bairu et al., 2011; Rai et al., 2011). This interval can be helpful in identification and therefore enabling elimination of transient or epigenetically resistant plants. Indeed, *in vitro* screening technique is based on the fact that (abiotic) stress resistance levels are related to photosynthesis and plant growth (Ashraf and Harris, 2013). Hence, somaclonal variants with increased resistance to an abiotic stressor often differ from mother plants (control) in terms of growth ability and showing sensitivity symptoms which can be detected through *in vitro* screening after plant regeneration from selected calli surviving selection against an abiotic stressor.

In a literature survey, *in vitro* screening has been found to be one of the most used methods to evaluate the relative resistance of regenerants to different abiotic stresses such as drought, salinity and heavy metals (Albiski et al., 2012; Gopal and Iwama, 2007; Santandrea et al., 1998; Zhang and Donnelly, 1997; Zhao et al., 2013). To assess the relative resistance of different plant lines or genotypes using this approach, equivalent plant materials, for example, nodal shoot cuttings of similar size, from each of the putative somaclonal variants as well as the mother plants were exposed to the stress factor under investigation spiked in a growth medium under the same culture conditions for a period of time varying from few weeks to two months dependent on the toxicity of the stress factor. Subsequently, the growth and development of injury symptoms in the putative somaclonal variants were evaluated and compared with the control (mother plantlets).

In vitro screening can be initiated using a variety of materials from putative somaclonal variants and the mother plants including shoot tips, single-node stem cuttings, root-tip segments, or microtubers (Gopal and Iwama, 2007). For instance, Santandrea et al. (1998) exposed 2-cm tobacco shoot tips with the same number of leaves as the starting materials to three concentrations [0.1 (control), 2 and 4 mM] of manganese (Mn) added to MS culture medium for six weeks. Afterwards, the plantlets were scored for six parameters including root and shoot lengths, fresh and dry weights, leaf number, and injury symptoms including necrosis or chlororsis. The results showed that 2 and 5 mM Mn treatments caused less growth inhibition and injures in the plantlets derived from *in vitro* selected putative somaclonal variants than control plantlets. In fact, this was the only report showing that *in vitro* selected HM-resistant variants grew better than the non-selected control plantlets in the presence of HMs using this *in vitro* screening approach.

Screening of *in vitro* selected plants can also be possible using hydroponic growth techniques. For instance, Nehnevajova et al. (2007) observed three different phenotypes of Indian mustard (*Brassica juncea*) with improved HM-resistance through screening different lines of putative somaclonal variant plants grown hydroponically in a growth chamber. Interestingly, some of them showed different responses to different HMs (Cd and Pb) (Nehnevajova et al., 2007). For example, exposure to 10 µM Cd resulted in biomass decrease of some lines, whereas 10 µM Pb treatment had positive effects on their growth. A hydroponic system was also recently used to screen tomato plants for resistance to 25 µM $CdCl_2$ (Piotto et al., 2014). However, application of hydroponic screening is not always desirable due to its relatively high costs, space requirement and possible complications from microbial interactions.

4.2. Aim and experimental approach

The main aim of the work described in this chapter was to identify the promising *in vitro*-generated potato lines with significantly improved Cd-resistance compared to the non-selected control (mother plants). To achieve this aim, at first, growth of all the lines regenerated from Cd-exposed calli was screened under *in vitro* conditions and compared with the control plantlets when they were grown in the presence of a relatively low or non-lethal Cd concentration. The lines which showed promising growth performance in the first screening round were selected for a further round of screening evaluating their survival under a Cd level that is normally lethal to the non-selected mother plants. Besides vegetative growth, the severity of visible injuries in the selected plant lines was also assessed.

4.3. Materials and methods

4.3.1. Plant materials and culture conditions

To identify plant lines with enhanced Cd resistance, their growth was compared to that of control plants in the presence of a relatively low, non-lethal Cd concentration (29 μM). For each plant line, shoot segments (3 cm each) of approximately the same size and with three leaves were excised from the middle of plantlets while the shoot tip and base were discarded. Afterwards, the 18 excised segments (one from each of the putative somaclonal variant plant lines) were randomly cultured in six jars (three segments in each jar) containing 50 mL of basal half-strength MS medium supplemented with 29 μM $CdCl_2$ for 10 days. Each of these cultures had six replicates. One control segment prepared in the same way as the variant lines was also co-cultured with the other segments from the plant lines in each jar (Fig. 29).

Figure 29. A culture of shoot segments of approximately the same size and with three leaves (the first round of screening).

The second round of screening was carried out with five lines selected based on the findings in the first screening round. Like the first round, shoot segments (3 cm each) of approximately the same size and with three leaves were excised from the middle of the plantlets of each line. The five excised segments as well as one control segment prepared in the same way as the variant lines were co-cultured in a jar containing 50 mL of basal half-strength MS medium supplemented with 218 μM $CdCl_2$ (the Cd concentration that was found to be lethal to potato plantlets in Chapter 3) for 20 days. This was carried out with six replicates.

All the media were supplemented with 3% (w/v) sucrose, adjusted to pH 5.8 with 1 M KOH or HCL, and solidified with 0.8% (w/v) agar. Afterwards, they were autoclaved at 121^{o}C under 103 kPa for 20 minutes. Culture tools including scalpels, forcepses and scissors, were wiped with 70% (v/v) ethanol and then their tips were dipped in a hot glass bead dry steriliser (Steri 250, Switzerland) for sterilisation. All the plantlets were grown inside 250-ml clear polycarbonate tissue culture jars (8.5 cm height and 6.5 cm diameter) in a growth room at 21$\pm$1^{o}C under continuous lighting (Sylvania Gro-Lux lamps 36W).

4.3.2. Assessment of Cd impacts on growth of cultures

At the end of both screening rounds, the plantlets were handled and harvested very carefully to minimise root breakages during plantlet removal from the solidified culture medium. The residual agar adhered to the roots were washed off using Milli-Q water before measuring the shoot and root lengths. Also, the number of newly developed leaves from each plantlet was counted. In the second screening round, visible injury symptoms including necrosis or chlorosis of each plantlet were also recorded. Afterwards, the fresh weights of the shoots and roots were determined separately. The shoot and root dry weights were also determined following drying in an oven (CONTHERM – CON8050) at 80°C for 48 hours.

4.3.3. Statistical analysis

All the experiments were carried out three times and there were six replicates in each treatment. Data were subjected to one way analysis of variance (ANOVA, $p \leq 0.05$) followed by comparison of the means using Duncan's multiple range test at 5% level of significance. The data were assessed using the statistical program SPSS version 19.0.

4.4. Results

In the first screening round, five lines namely 3, 6, 7, 9 and 11 exhibited significantly stronger growth abilities than the control in the presence of a low, non-lethal Cd concentration (Table 15). However, among them, line 11 showed the best performance with respect to the chosen parameters, followed by lines 9 and 7 which both had almost the same growth abilities. Generally, the shoots of these five lines of plantlets developed five to seven more leaves than control plantlets. Furthermore, their root and shoot lengths were about 2.5 and 6-8 cm longer than the control, respectively (Table 15). With respect to almost all the parameters scored, the other 13 lines were similar to those of control plants (Table 15). Furthermore, the plantlets of the screened lines and the control did not show any visible injury symptoms and almost all of them seemed healthy after ten days of culture. Similarly, no symptom of dehydration, chlorosis, or necrosis was seen in two lettuce (*Lactuca sativa*) cultivars grown in the presence of 15 μM $CdCl_2$ (Zorrig et al., 2013). Short-term (one week) exposure to 20 or 40 μM Cd could even stimulate shoot and root growth in *Pfaffia glomerata* plantlets (Skrebsky et al., 2008).

To evaluate the five best performing lines further, they were subjected to the second round of screening under lethal Cd stress. The results of this screening round revealed that lines 11 and 9 not only survived under the lethal Cd stress level (218 μM) for 20 days, but interestingly also exhibited increased growth (Table 16; Fig. 30A&B). The average root length of these two lines was more than eight cm (Table 16) (Fig. 30D). In contrast, the control shoot cultures failed to form roots (Fig. 30E). Likewise, shoot elongation was almost completely prevented in control shoot cultures, whereas the shoots of the plantlets in lines 11 and 9 were at least four cm long. Moreover, at least six new leaves were formed in the shoots of these two lines, while no new leaf was formed in the control shoot cultures (Table 16).

Apart from very few necrotic areas on the shoots, interestingly there was no major visible injury symptom on the plantlets of these two lines, whereas almost all the leaves in the control shoot cultures became completely necrotic in response to 218 μM Cd (Fig. 30C&E). There was no difference in the fresh weights of the roots and shoots of these two lines but the dry root and shoot weights of line 9 were 20 and 10 percent lower than those of line 11, respectively (Table 17).

Under low, non-lethal Cd stress, line 7 grew as well as line 9, but in response to lethal stress, line 9 grew better than line 7 (Table 16). After 20 days of culture in the presence of the lethal Cd level, the shoot lengths of lines 3, 6 and 7 were approximately double those of the control (Table 16). While the control shoot cultures failed to form leaves or roots, the roots of these three lines were four to seven cm long and one to three new leaves were formed. However, necrotic tissues were observed all along their shoots, particularly the basal ends, as well as the top portion of their roots while leaf necrosis was rarely found (Fig. 31). The average fresh and dry weights of the roots and shoots of these three lines were about two and four times less than those of line 11, respectively (Table 17).

Table 15. Effects of 29 μM Cd treatment on vegetative growth in 18 somaclonal plant lines after 10 days of culture.

Parameter / Line	Root length (cm)	Shoot length (cm)	Number of new leaves formed
Control	3.4±0.65^{cd}	4.0±0.21^{c}	1.3±0.12^{e}
1	3.4±0.43^{cd}	4.1±0.34^{c}	1.2±0.21^{e}
2	3.2±0.73^{d}	4.2±0.11^{c}	1.4±0.02^{e}
3	9.9±0.10^{b}	6.4±0.56^{b}	6.9±0.04^{b}
4	3.9±0.31^{c}	4.4±0.07^{c}	1.7±0.08^{de}
5	3.3±0.37^{cd}	4.3±0.24^{c}	1.1±0.10^{e}
6	10.2±0.22^{b}	6.0±0.43^{b}	6.0±0.14^{c}
7	11.1±0.17^{a}	7.9±0.66^{ab}	7.3±0.09^{ab}
8	3.0±0.48^{d}	4.6±0.28^{bc}	2.2±0.20^{d}
9	11.0±0.93^{a}	8.0±0.33^{ab}	7.4±0.12^{ab}
10	3.1±0.12^{cd}	4.2±0.44^{c}	1.9±0.18^{d}
11	11.3±0.14^{a}	8.5±0.18^{a}	7.9±0.04^{a}
12	3.1±0.19^{cd}	4.2±0.22^{c}	1.5±0.11^{de}
13	3.2±0.91^{cd}	4.1±0.67^{c}	1.5±0.32^{de}
14	3.4±0.80^{cd}	4.4±0.90^{c}	1.2±0.41^{e}
15	3.5±0.13^{cd}	4.2±0.66^{c}	1.4±0.07^{e}
16	3.1±0.59^{cd}	4.1±0.10^{c}	1.6±0.60^{de}
17	3.2±0.12^{cd}	4.1±0.12^{c}	1.4±0.44^{e}
18	3.0±0.09^{d}	4.2±0.21^{c}	1.2±0.56^{e}

- Means within a column having the same letter are not significantly different by Duncan's multiple range test ($p<0.05$).

Table 16. Effects of 218 μM Cd treatment on vegetative growth and induction of injury symptoms in five lines selected from the first screening round after 20 days of culture.

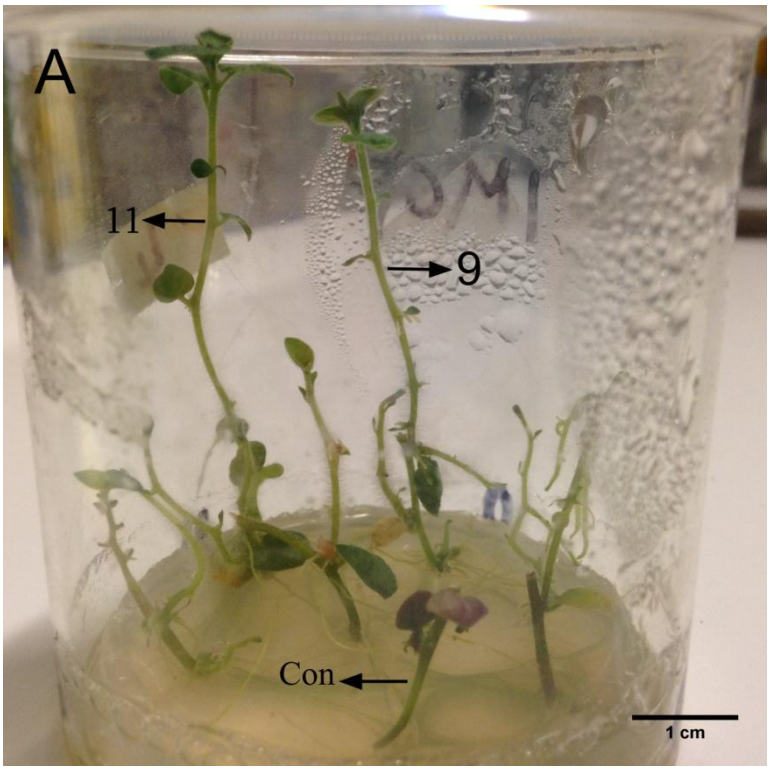
Parameter / Line	Root length (cm)	Shoot length (cm)	Number of new leaves formed	Injury severity
Control	0 ± 0.0^{d}	2.2 ± 0.01^{d}	0 ± 0.0^{e}	Major
3	7.1 ± 0.11^{b}	4.5 ± 0.16^{b}	3.3 ± 0.14^{b}	Mild
6	6.5 ± 0.62^{bc}	4.3 ± 0.13^{bc}	1.0 ± 0.33^{d}	Mild
7	4.3 ± 0.41^{c}	4.9 ± 0.29^{b}	2.2 ± 0.74^{c}	Mild
9	8.1 ± 0.80^{a}	6.1 ± 0.47^{ab}	6.4 ± 0.36^{ab}	Minor
11	8.5 ± 0.10^{a}	6.8 ± 0.70^{a}	7.1 ± 0.69^{a}	Minor

- Means within a column having the same letter are not significantly different by Duncan's multiple range test ($p<0.05$).

Table 17. Effects of 218 μM Cd treatment on fresh and dry weights of roots and shoots in 5 selected lines from the first screening round after 20 days of culture.

Parameter / Line	Root F.W. (g)	Root D.W. (g)	Shoot F.W. (g)	Shoot D.W. (g)
Control	0.0 ± 0.00^{d}	0.0 ± 0.000^{e}	0.09 ± 0.01^{c}	0.008 ± 0.005^{d}
3	0.20 ± 0.06^{b}	0.02 ± 0.002^{bc}	0.16 ± 0.02^{bc}	0.016 ± 0.007^{c}
6	0.18 ± 0.09^{b}	0.016 ± 0.007^{c}	0.17 ± 0.02^{bc}	0.012 ± 0.009^{c}
7	0.11 ± 0.04^{c}	0.009 ± 0.008^{d}	0.24 ± 0.00^{b}	0.015 ± 0.008^{c}
9	0.26 ± 0.02^{a}	0.027 ± 0.005^{b}	0.33 ± 0.05^{ab}	0.038 ± 0.006^{b}
11	0.30 ± 0.09^{a}	0.038 ± 0.002^{a}	0.37 ± 0.02^{a}	0.047 ± 0.002^{a}

- Means within a column having the same letter are not significantly different by Duncan's multiple range test ($p<0.05$).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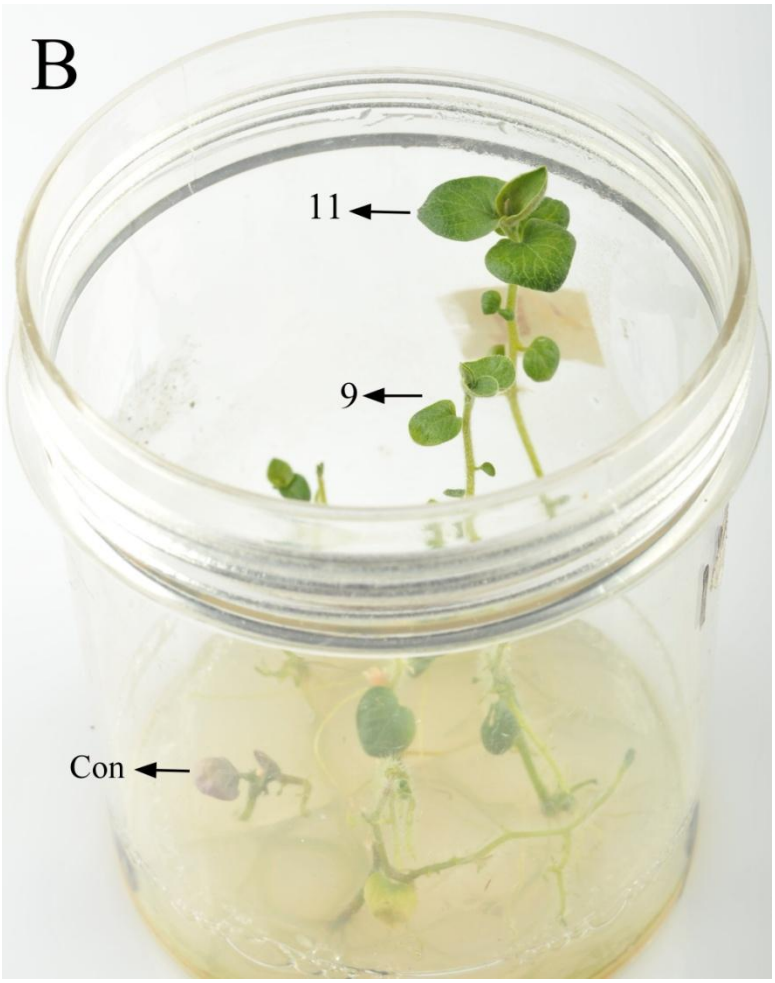
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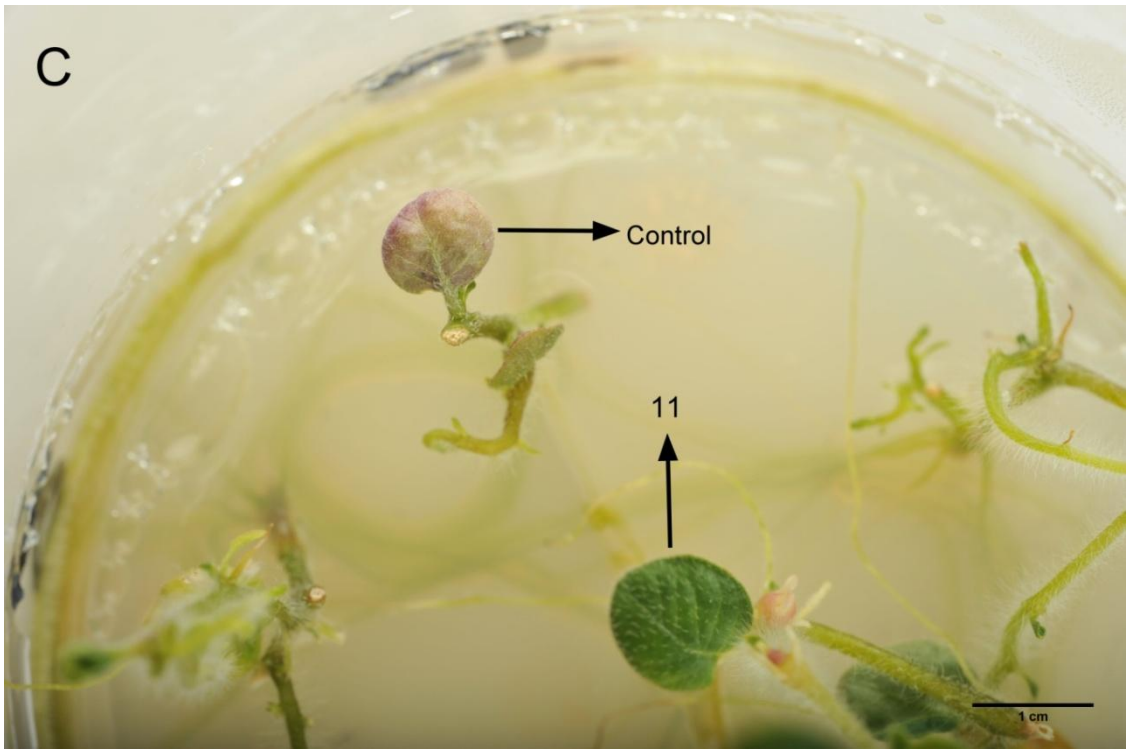


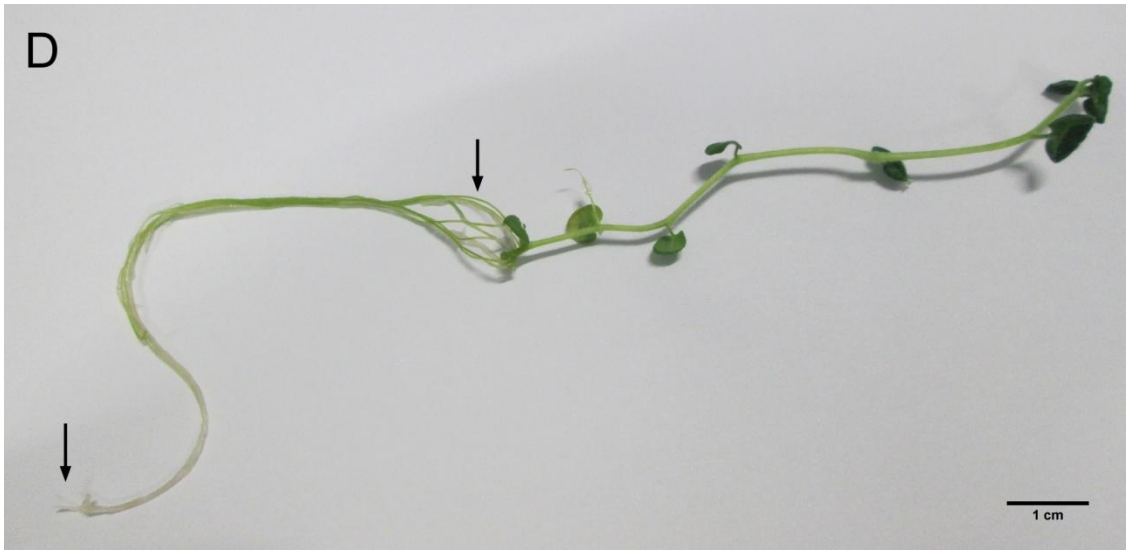


Figure 30. Outstanding vegetative growth in two *in vitro*-generated plant lines, namely 11 and 9, in the presence of the lethal Cd concentration (218 µM Cd) compared to control. (A&B) Comparison of shoot heights from 90^{o} and 45^{o} views, respectively; (C) a healthy green leaf of a line 11 plantlet and a necrotic leaf of a control shoot; (D) a root of a line 11 plantlet; (E) lack of root formation in a control shoot segment.

Figure 31. Necrotic (brown) tissues observed along a shoot of line 3 plantlet, particularly its basal end, and the very top portion of the root near the shoot-root junction.

4.5. Discussion

The present *in vitro* screening programme comprising an initial low or non-lethal Cd treatment followed by exposing the few selected ones to a second round of a lethal Cd treatment was successful in assessment of the differential growth abilities of 18 *in vitro* generated lines exposed to Cd stress. In the first round, some lines were distinguishable from the others which showed no Cd-resistance improvement compared to the control. These lines were probably "escape" plants regenerated from the Cd-selected calli. High escape frequency is one of criticisms to *in vitro* selection system. However, application of fast-screening method for eliminating escaped plants has been preferred to aiming for an escape-free selection system (Hell, 2003). Vyver et al. (2013) also reported 20 percent escape rate of non-herbicide tolerant sugarcane plantlets in an *in vitro* regime. Thus, this confirms the importance of the *in vitro* screening step post plant regeneration from Cd-selected calli.

The key growth parameters, particularly root and shoot dry weights, in this study of assessment of the *in vitro*-generated lines, were found to be useful in identification of the most promising lines with improved Cd resistance. Dry matter measurement was also promising in screening of *Nicotiana* species and flax varieties for tolerance to Mn and Cd/Zn respectively (Santandrea et al., 1998; Smykalova et al., 2010). Indeed, this was the first time that HM resistance was assessed in a crop species (potato) through the *in vitro* approach used here. In sweet potato, this approach (*in vitro* screening) was previously employed for assessment of salt tolerance in *in vitro* selected lines by comparing root numbers and lengths. (He et al., 2009). Regarding the importance of uniformity of culture conditions in which the lines were compared, in the second round of screening, it is of interest to note that all of the five selected lines from the first round were co-cultured and grown on the

same Cd-containing culture medium in a same jar. This condition has not been performed previously.

In potato, application of *in vitro* screening was found to be effective for evaluation of resistance to other abiotic stresses including heat, drought and salinity (Albiski et al., 2012; Gopal and Iwama, 2007; Zhang and Donnelly, 1997). One of the reasons can be the high amenability of potato to tissue culture techniques (Gopal and Iwama, 2007). As it has been mentioned in Chapter 3, Iwa is one of the most responsive potato cultivars to *in vitro* growth studies. Using *in vitro* screening, Gopal and Iwama (2007) could distinguish three lines of Iwa differing in terms of drought resistance level.

Application of more than one concentration of a screening factor, has been highly recommended in many previous studies (Rahman et al., 2009; Santandrea et al., 1998; Zhang and Donnelly, 1997). For instance, Santandrea et al. (1998) evaluated Mn tolerance of tobacco regenerants by treating them with 2 and 5 mM Mn under *in vitro* conditions. This clearly confirmed their increased Mn tolerance compared to the control plantlets.

Here, line 7 was not only found to be different from line 9 in response to the low Cd level, but in the second round of screening under lethal Cd stress line 7 was found to be less resistant than line 9 to the Cd treatment. Therefore, application of more than one concentration of the stress factor under study would be helpful for distinguishing the *in vitro*-generated plant lines.

The results of the present study showed that five lines of the 18 putative somaclonal variant lines grew relatively better under Cd stress than control plantlets. This validates the success of the *in vitro* selection programme (described in Chapter 3) in improvement of Cd resistance in Iwa potato. Among these five lines, only lines 11 and 9 were particularly promising for further work due to their outstanding growth abilities under lethal Cd stress.

However, great shoot and foliage growth do not necessarily correlate with a high yield (Zhang and Donnelly, 1997). The dry weight measurements further differentiate between line 11 and line 9 in terms of the lower biomass decrease in line 11 compared to line 9 in response to the high level of Cd.

Apart from the mentioned advantages, the lack of any major visible injury on the plant parts including the root, shoot and leaf of line 11 plantlets was very interesting while they were exposed to a Cd level lethal to control plantlets.
As shown in this study and other similar works, the root was found to be a sensitive plant organ to severe abiotic stress (Rahman et al., 2009; Santandrea et al., 1998). Examples include reduced root formation and developmental arrest in control tobacco and potato plantlets in response to exposure to high Mn and Cd concentrations, respectively (Santandrea et al., 1998). Likewise, a high level of salinity (100 mM NaCl) arrested *in vitro* root development in three potato cultivars (Rahman et al., 2009). The meristematic zone of the roots has been suggested as a main target of metal toxicity (Santandrea et al., 1998). All these can be related to the key roles of the roots in regulation of Cd translocation from the culture medium to the above-ground organs, particularly the leaf. However, for a better understanding of the mechanism(s) involved, further studies such as biochemical and microscopic studies on Cd-stress resistant and control plants are required, particularly at the low, non-lethal Cd level.

Chapter 5 – Biochemical and Microscopic Studies on the *In vitro* Selected Cd-Resistant Potato Lines

5.1. Introduction

Heavy metal resistant plants developed through *in vitro* breeding may have different resistance mechanisms or desirable attributes including enhanced tolerance, minimised uptake or translocation of the metal under study (Fig. 32) (Clemens, 2001; Zhu et al., 2007). As the first defence line, plant roots may avoid excess metal uptake by limiting metal ions to the apoplast, binding them to the cell walls or cellular exudates restricting metal transport from the roots to aerial parts (Fig. 32a-c). Heavy metal excluder plants are often good at this strategy, particularly through cell walls with increased metal binding capacity (Gallego et al., 2012; Hall, 2002). To address the adverse impacts of metal ions which could get through the first defence line and have already accumulated in the cells, exploitation of many other detoxification strategies including compartmentalisation and complexing with metal-binding peptides or organic ligands is possible (Fig. 32d-f). Cadmium ions often chelate preferentially to sulphur ligands rather than organic ligands (Salt et al., 2002). However, toxic metal-induced oxidative stress to the cells can still result which plants need to counteract by activating the respective defence system called antioxidative system (Fig. 32g).

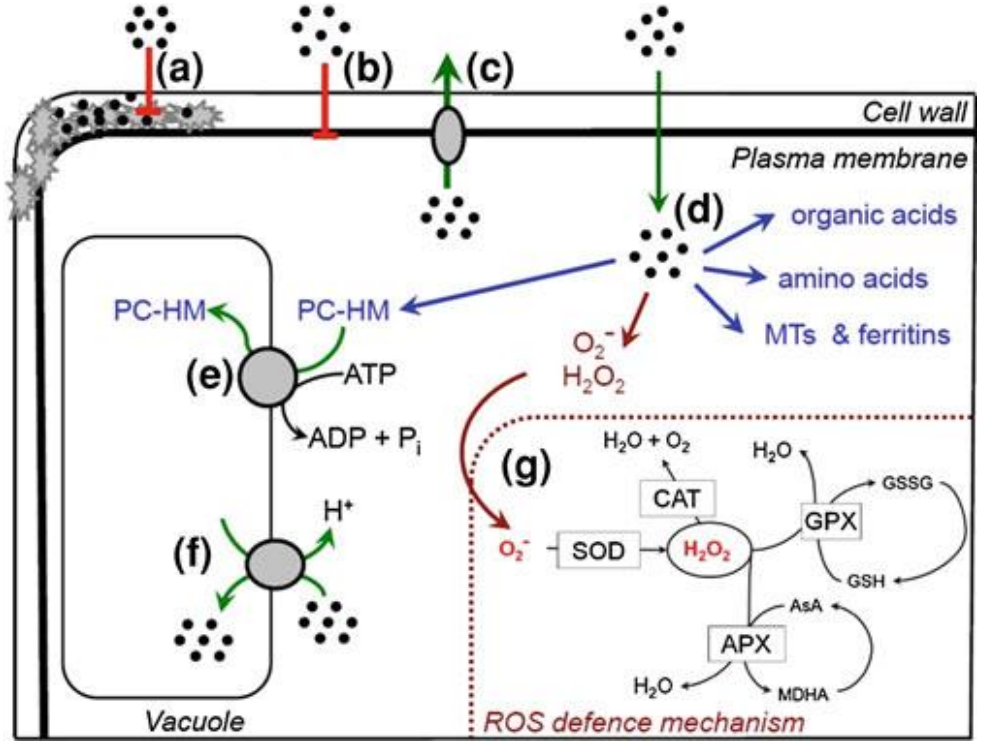


Figure 32. Heavy metal defence mechanisms in plants: (a) binding metal ions to the cell wall and root exudates; (b) inhibitory role of plasma membrane on metal ion influx; (c) pumping of metal ions into apoplast; (d) metal chelating; (e) transfer of metal-ligand complexes to vacuole through tonoplast; (f) compartmentalisation of metal ions into vacuole via specialised antiporters; (g) antioxidative defence mechanism arisen in response to induced ROS (Hall, 2002).

It is clear that HM-resistant somaclones do not necessarily base on the mechanisms of traditional plant breeding as the nature of somaclonal variation is largely based on chance mutation (Predieri, 2001). However, if plants developed using *in vitro* techniques based on somaclonal variation possess the sought-after trait, it means that an *in vitro* breeding programme and the associated aim has been successful. This achievement has more importance when it comes to food crop species. For instance, increased HM-accumulation in above-ground parts of plants is a trait of concern for food crop species threatening food safety (Van der Ent et al., 2013), while it is desirable for hyperaccumulator species intended for cleaning up HM-contaminated soils (phytoremediation). However, every *in vitro* breeding programme could lead to generation of undesirable somaclones as well as somaclones of interest, or even other mutations with unknown attributes. For instance, among the plant regenerants from an *in vitro* breeding experiment of Indian mustard (*Brassica juncea* L.), plant lines with either reduced or enhanced metal accumulation were found. On the other hand, a big group of plant regenerants showed no change in the HM accumulation trait (Nehnevajova et al., 2007).

Understanding metal resistance mechanism in the new HM-resistant plants developed from breeding is fundamental before introducing them as new cultivars (Baisakhi et al., 2003). This understanding can bring high values to particularly the somaclonal variants, having advantages over transgenic plants (GMOs) in terms of more acceptability to the public (Predieri, 2001). For this aim, different studies including microscopic, biochemical and molecular studies have been suggested (Clemens, 2001). These studies can be even more beneficial when an abiotic stress is not that strong to affect plant growth abilities or morphological characters making *in vitro* screening approach unable to identify resistant somaclones initially.

Previous studies showed that alternations in photosynthesis, free radical formation, activities or content of enzymatic or non-enzymatic antioxidants are some of the key symptoms of Cd stress in the plants (Cherif et al., 2012; Marmiroli et al., 2013; Wang et al., 2014a). Therefore, they could be used as biomarkers to help to study Cd-resistance mechanisms involved.

Comparison of intra-cellular localisation of Cd in Cd-tolerant and non-tolerant plants can also be fundamental in obtaining a better understanding of Cd tolerance mechanism (Mullins, 1987). In relation to this aim, study of metal uptake into the leaf is important (Cosio et al., 2006; Vollenweider et al., 2006). However, similar studies in other plant parts were also valuable, particularly in the roots playing regulatory roles in metal uptake from the soils (Belleghem et al., 2007). To visualise the sub-cellular distribution of Cd, the most used technique was electron microscopy. Application of other techniques such as X-ray micro-analysis was less common due to their greater interference with other cations (e.g. K) and relatively poor detection sensitivity limiting Cd detection (Cosio et al., 2006; Seregin and Ivanov, 2001).

5.2. Aims

This chapter aimed to obtain some basic understanding of potential Cd resistance mechanisms in potato lines selected previously through the *in vitro* screening (in Chapter 4). Thereby, biochemical and electron microscopic studies were employed to assess these lines compared with those of the mother plant (control). Electron microscopic study can reveal intra-cellular localisation of Cd ions in the plant parts with implication for the potential of Cd resistance mechanisms at the sub-cellular level. In addition, it can uncover damages to intra-cellular structures and organelles caused by Cd-imposed oxidative stress. The extent of this stress was estimated by analysing the ROS and antioxidative enzyme profiles of the plants.

5.3. Materials and methods

One-week-old plantlets from each of the lines 9 and 11 as well as control stock plants were randomly chosen and cultured in the same jar containing 50 mL half-strength basal MS medium supplemented with 0, 29 and 218 μM $CdCl_2$ for 7 days. This was carried out with four replicates. All the medium preparation and sterilisation processes as well as culture conditions were as described in Chapter 4. After one week of culture, the plantlets were harvested and then their roots and leaves were separated for biochemical studies including determination of lipid peroxidation, hydrogen peroxide level, activities of antioxidative enzymes, and contents of glutathione, proline and total chlorophyll. The line which showed relatively higher Cd-resistance (line 11) and the control were subjected to microscopic study.

5.3.1. Lipid peroxidation

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) production using the thiobarbituric acid (TBA) method (Demiral and Türkan, 2005). To extract MDA, 0.5 g fresh weight of roots or leaves was ground in liquid nitrogen using a mini pestle and mortar and then homogenised in 2.5 mL of ice-cold 0.1 % (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 g for 15 min at 4^{o}C. Subsequently, 4 mL of 20 % (w/v) TCA supplemented with 0.5 % TBA was added to each 1 mL aliquot of the supernatant. Afterwards, the mixture was heated at 95^{o}C for 30 min, cooled quickly on an ice-bath, and centrifuged for 15 min at 12,000 g. Finally, the absorbance was measured at 532 nm and then it was corrected by subtracting the absorbance at 600 nm (molar absorptivity of 155 mM^{-1} cm^{-1} was used in determination of MDA concentration).

5.3.2. Hydrogen peroxide level

Hydrogen peroxide (H_2O_2) level was determined according to Singh et al. (2006). Thereby, 0.5 g fresh weight of roots or leaves was ground in liquid nitrogen using a mini pestle and mortar and then homogenised in 5 ml of ice-cold 0.1 % (w/v) trichloroacetic acid (TCA). The homogenate was centrifuged at 12,000 g for 15 min at 4^{o}C. Afterwards, 0.5 ml of the supernatant was added to 0.5 ml of 10 mM potassium phosphate buffer (pH 7), and 1 mL of 1 mM potassium iodide (KI). Hydrogen peroxide content was measured by reading the supernatant absorbance at 390 nm by using a spectrophotometer (BIO RAD-Smartspec^{TM} Plus).

5.3.3. Antioxidative enzyme assays

5.3.3.1. Sample extraction

Sample extraction was carried out according to a modified method of Martins et al. (2014). One gram (fresh weight) roots or leaves were ground in liquid nitrogen using a mini pestle and mortar, and homogenised in 1 ml of 50 mM potassium phosphate buffer (pH 7.4) containing 0.1 mM Na_2EDTA, and 2% (w/w) insoluble polyvinyl polypyrrolidone (PVP). The homogenate was then centrifuged at 12,000 g for 15 min at 4^{o}C. Afterwards, the supernatant was filtered using VIVASPIN 500 (5000 MWCO) ultrafiltration units. Extraction of ascorbate peroxidise (APX) was carried out separately using the same method, except addition of 5 mM ascorbate to the potassium phosphate buffer. The activities of the antioxidative enzymes were determined according to the following procedures.

5.3.3.2. Superoxide dismutase

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed according to the method of Martins et al. (2014) by measuring an increase in absorbance at 550 nm for 2 min in 100 mM potassium phosphate buffer (pH 7.6) containing 0.05 mM ferricytochrome-C, 0.5 mM xanthine, 0.1 mM EDTA and xanthine oxidase. The enzyme activity was determined as the enzyme quantity required to inhibit the reduction of ferricytochrome-C by 50 percent, per min and mL.

5.3.3.3. Catalase

Catalase (CAT; EC 1.11.1.6) activity was determined according to the method of Martins et al. (2014), by measuring a decrease in absorbance at 240 nm for 2 min in 50 mM phosphate buffer (pH 7.0) containing 10 mM of hydrogen peroxide. The enzyme activity was defined as the consumption of l μmol H_2O_2 per min and mL.

5.3.3.4. Guaiacol peroxidise

Cadmium impacts on guaiacol peroxidise (GPX; EC 1.11.1.7) activity was quantified as described by Ranieri et al. (1997). Briefly, it was measured in 1 mL assay mixture consisting of potassium phosphate buffer (0.05 M, pH 6.9), guaiacol (Sigma, St. Louis, USA) as substrate, and enzyme extract. Enzyme extract (root or leaf supernatant) was omitted from the control assay mixture. The absorbance at 470 nm was measured spectrophotometrically after incubation at 23^{o}C for 30 seconds. One unit of enzyme activity was defined as that which brought about a change of 0.01 absorbance unit per minute (Ranieri et al., 1997).

5.3.3.5. Ascorbate peroxidase

Ascorbate peroxidase (APX; EC 1.11.1.11) was determined according to the method of Sharma and Dubey (2004) in a reaction mixture: 50 mM phosphate buffer (pH 7.0) containing 0.25 mM ascorbic acid and 0.3 mM H_2O_2 followed by measuring a decrease in absorbance of 290 nm. One unit activity of APX is defined as the amount of enzyme which can oxidize 1 μMol ascorbic acid per minute.

5.3.3.6. Glutathione reductase

Glutathione reductase (GR; EC 1.6.4.2.) activity was estimated according to Phang (2010). The reaction mixture contained 150 μL of 100 mM sodium phosphate buffer (pH 7.6), 10 μM NADPH (10mg mL^{-1}; 12 mM), 0.1 mM DTNB, and 50 μL enzyme extract (dH2O as control). The reaction was started by adding 10 μL GSSG (1 mg mL^{-1}; 3.25 mM) followed by reading the absorbance at 415 nm for 3 min at 30 second intervals. The increase rate in A_{415} per min was calculated and then based on the change in A_{415} using standard wheat germ GR, a regression analysis was used to create a standard curve for GR activity. Specific GR activity was interpolated against the standard curve and expressed as unit mg^{-1} FW.

5.3.4. Proline content

Estimation of free proline content was carried out according to Bates et al. (1973). Briefly, root or leaf samples (0.5 g) were homogenised in 3% (w/v) sulphosalycylic acid followed by filtering through a filter paper. Afterwards, the filtrate was mixed with acid ninhydrin and glacial acetic acid and then heated at 100^{o}C for 1 hour. The reaction was terminated by cooling on an ice bath. Subsequently, the mixture was extracted with toluene, and the absorption of the chromophore was read at 520 nm. Proline concentration was determined using a calibration curve and expressed as μMol proline mg^{-1} FW.

5.3.5. Chlorophyll content

The relative chlorophyll content was determined by using a Chlorophyll Meter (SPAD-502; Minolta, Japan) measuring the absorbance of the leaf in blue (400-500 nm) and red (600-700 nm) regions (Huang et al., 2013). The average of six readings, taken around the mid of the leaf disc, was assumed as the chlorophyll content of the leaf.

5.3.6. Glutathione concentration

To determine glutathione concentration, 5g of plant material was homogenised in 100 mL TCA (trichloroacetic acid) and 0.125 mM phosphate buffer (pH 6.3) supplemented with 6.3 mM EDTA. Afterwards, the homogenates were centrifuged at 10,000 g for 10 min at 4^{o}C. Glutathione concentration was determined by calculating the linear changes in absorbance of the reaction mixtures measured at 412 nm, and expressed as μMol GSH g^{-1} FW.

5.3.7. Transmission Electron Microscopy

The TEM methods outlined below are according to those of Jarvis (2001).
For this study, one-week-old plantlets of line 11 and control were grown on medium containing 0 and 29 μM $CdCl_2$ for 7 days. Afterwards, random two-week-old plantlets from each were selected followed by dissecting their leaves and roots. Root tissues located about 2 mm above the root tips were cut into sections with thickness of about one mm. Leaves were firstly cut into three equal pieces using a sharp scalpel and then the mid parts were used for microscopy. Afterwards, the dissected tissues were immediately fixed overnight in 0.1 M sodium phosphate buffer (pH 7.4) containing 4 % glutaraldehyde (v/v) and 1 % sodium sulphide (w/v) at room temperature under partial vacuum. Afterwards, the samples were post-fixed in 2 % OsO_4 for 2 hours at room temperature. The post-fixed samples were dehydrated in an ascending series of acetone (20, 40, 60 and 80 %, 10 min each) and finally 100 % acetone (15 min) for three times. The fixation and dehydration stages were carried out in a fume hood.

To infiltrate the samples in Spurr's resin, firstly, one part of Spurr's resin and two parts of 100% acetone were added to samples in vials and then placed on a slow rotating platform overnight. The second embedding step was carried out with three parts of Spurr's resin and one part of 100% acetone followed by rotating for three hours using the same platform. Afterwards, samples were transferred from vials to shallow plastic caps (by using a loop) containing 100% Spurr's resin, placed in glass Petri dishes, and heated in an oven at 70°C overnight. Subsequently, specimens were cut from resin blocks and glued to stubs (empty pill capsules filled with hardened epoxy resin). These were set overnight at room temperature before the stubs were trimmed to obtain desired tissue sections. Afterwards, the sections were mounted in an ultramicrotome (Leica ULTRACUT UCT, Germany*)* to be cut into approximately 90-100 nm

ultra-thin sections. Finally, ultra-thin sections were viewed with a transmission electron microscope (FEI Morgagni 268D, USA).

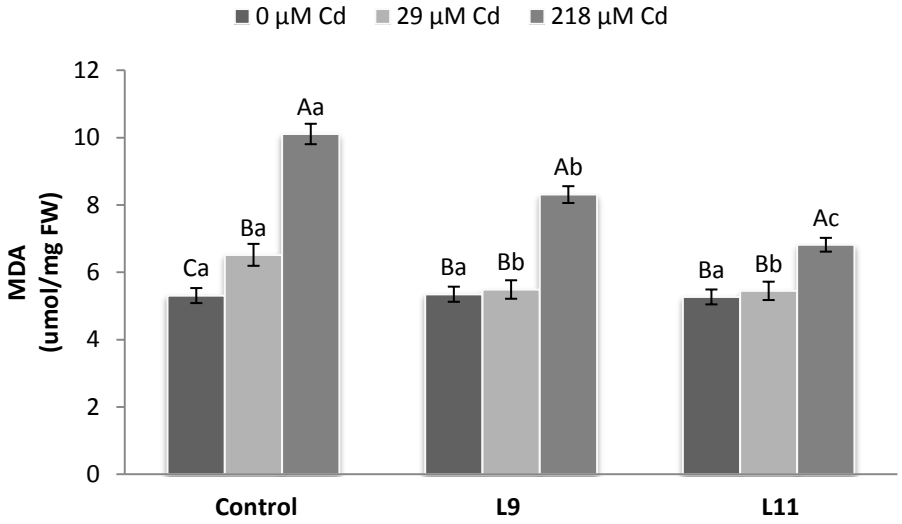
5.3.8. Statistical analysis

All the experiments were carried out twice and every independent treatment had four replicates. Data were subjected to one way analysis of variance (ANOVA, $p \leq 0.05$) following by comparison of the means using Duncan's multiple range test at 5% level of significance. The statistical program SPSS, version 19.0 was used.

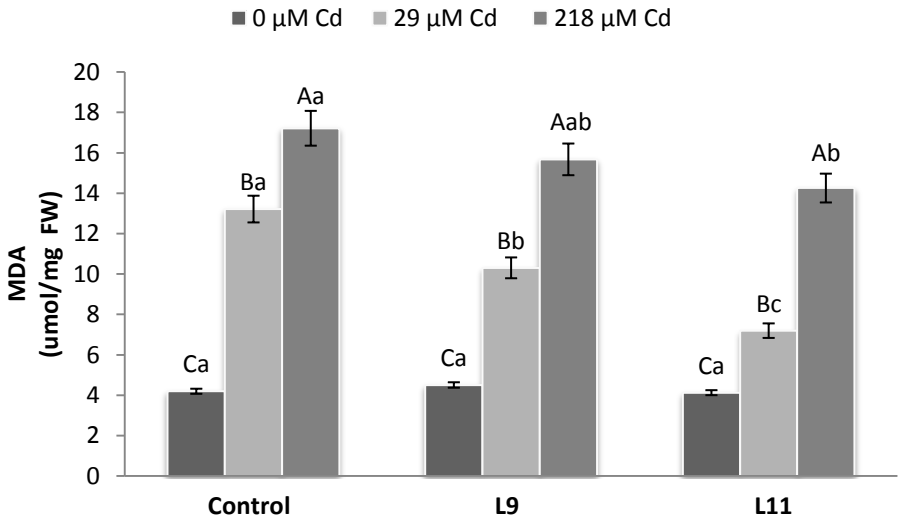
5.4. Results

5.4.1. Lipid peroxidation

Lipid peroxidation rates based on measurements of MDA levels in the leaves and roots of line 9 and 11 (herewith referred to as L9 and L11) plantlets were compared to control plantlets. In response to Cd exposure, generally, MDA levels in the roots were higher than the leaves in all the treatments (Fig. 33A&B). There was no significant difference in root or leaf MDA levels among both lines and control plantlets grown in the absence of Cd. Treatment with 29 μM Cd caused a significant increase (more than one unit over the level when grown in the absence of Cd) in control leaf MDA level, while did not bring any change in L9 or L11 leaf MDA level (Fig. 33A). In contrast, exposure to 29 μM Cd significantly increased MDA levels in control roots as well as those of L9 and L11. However, the results also showed that L11 experienced a lower rate of lipid peroxidation in the roots than the L9 and control (Fig. 33B). Addition of 218 μM Cd to growth media significantly increased lipid peroxidation in the leaves and roots of both lines as well as control (Fig. 33A&B). However, leaves of L11 contained the lowest MDA level compared to L9 and control, whereas the highest MDA level was found in control leaves. There was no significant difference in root MDA content between L9 and control, whereas L11 roots contained the lowest MDA content (Fig. 33B).



(A)

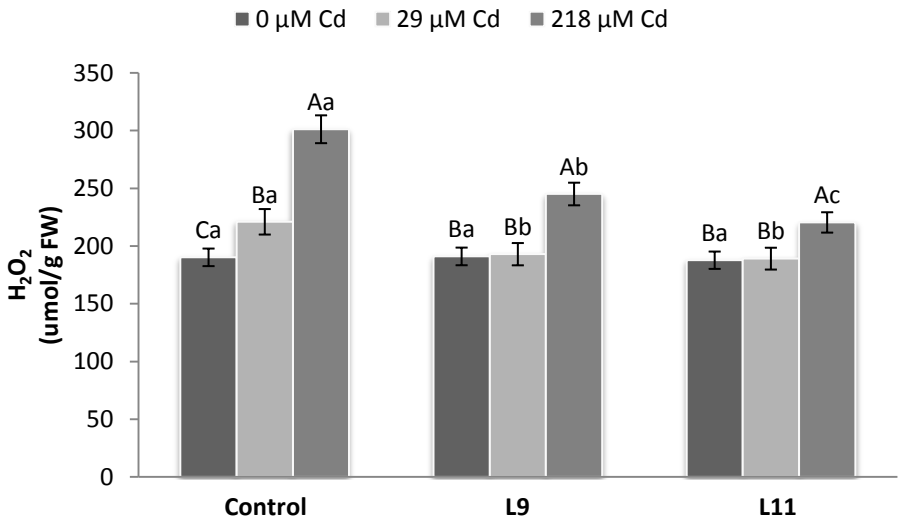


(B)

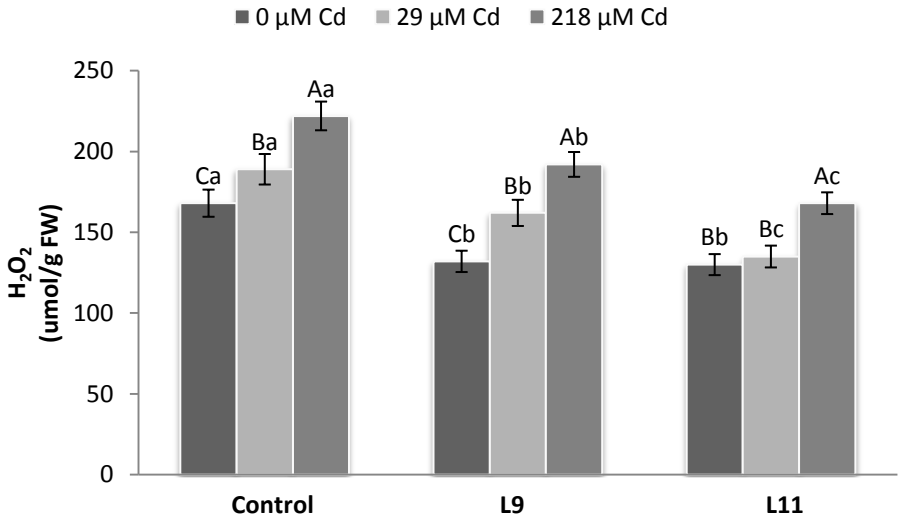
Figure 33. Monodehydroascorbate (MDA) level in (A) the leaves and (B) roots of control, L9 and L11 plantlelts exposed to 0, 29 and 218 µM Cd for 7 days. Upper and lower case letters are used for comparison among different treatments for the same line, and among different lines for the same treatment, respectively. Means with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$). The vertical bars show standard errors.

5.4.2. Hydrogen peroxide level

Basically, H_2O_2 level was noticeably higher in the leaves than roots (Fig. 34A&B). In the absence of Cd in the growth medium, H_2O_2 level in the leaves of L9 or L11 and control were at the same level (187-191 μmol g^{-1} FW) (Fig. 34A). In contrast, in the roots of L9 and L11 grown in the absence of Cd, the hydrogen peroxide levels were 132 and 130 μmol g^{-1} FW, respectively, which were significantly lower than control (168 μmol g^{-1} FW) (Fig. 34B). The content of H_2O_2 in leaves of control plantlets grown in 29 μM Cd-containing media was enhanced up to 221 μmol g^{-1} FW in comparison to growth in the absence of Cd, while no change in H_2O_2 level was seen in the leaves of L9 or L11 plantlets (Fig. 34A). In the roots, this change (increase in hydrogen peroxide level in response to 29 μM Cd compared to growth in the absence of Cd) was also significant in L9 although the enhanced level (162 μmol g^{-1} FW) was still significantly lower than that in control (189 μmol g^{-1} FW) (Fig. 34B). Exposure of the plantlets to 218 μM Cd brought significant increases in hydrogen peroxide levels in the roots or leaves of control, lines 9 and 11. The highest and lowest levels of hydrogen peroxide were recorded for control and L11, respectively (Fig. 34A&B).



(A)

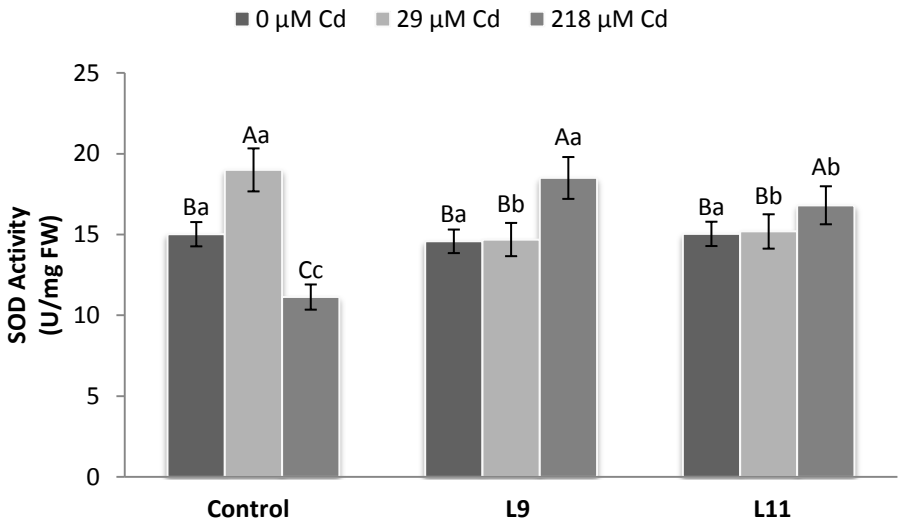


(B)

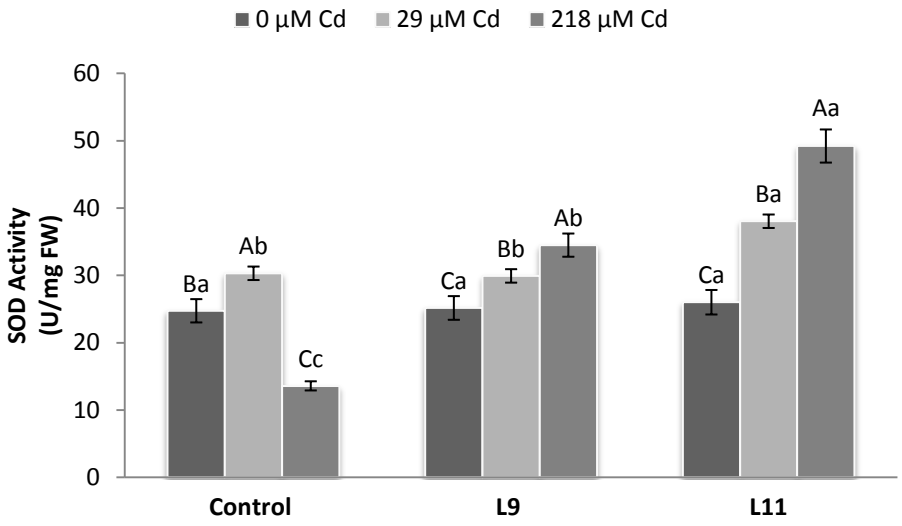
Figure 34. Hydrogen peroxide (H_2O_2) level in (A) the leaves and (B) roots of control, L9 and L11 plantlelts exposed to 0, 29 and 218 µM Cd for 7 days. Upper and lower case letters are used for comparison among different treatments for the same line, and among different lines for the same treatment, respectively. Means with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$). The vertical bars show standard errors.

5.4.3. Superoxide Dismutase (SOD)

The activity of SOD was basically higher in the roots than leaves (Fig. 35A&B). In the absence of Cd in the growth media, SOD activity in either the roots or leaves did not differ among the lines and control. Supplementation of the growth media with 29 μM Cd caused significant increases in SOD activity in the roots of L9 and L11 as well as control (Fig. 35B). However, the increase in L11 was more pronounced than those in L9 and control. On the other hand, in the leaves, this only enhanced control SOD activity (four units) and did not affect the enzyme activity in L9 or L11 (Fig. 35A). In response to the high Cd concentration (218 μM), L9 and L11 showed completely different SOD activities in both the roots and leaves compared to the control. The enzyme activity was significantly reduced in leaves and roots of control plantlets, whereas that was enhanced in those of L9 and L11. Comparison between these two lines showed that L9 was better at activating leaf SODs, whereas SODs in the roots of L11 showed higher activity than in the roots of L9 (Fig. 35A&B).



(A)

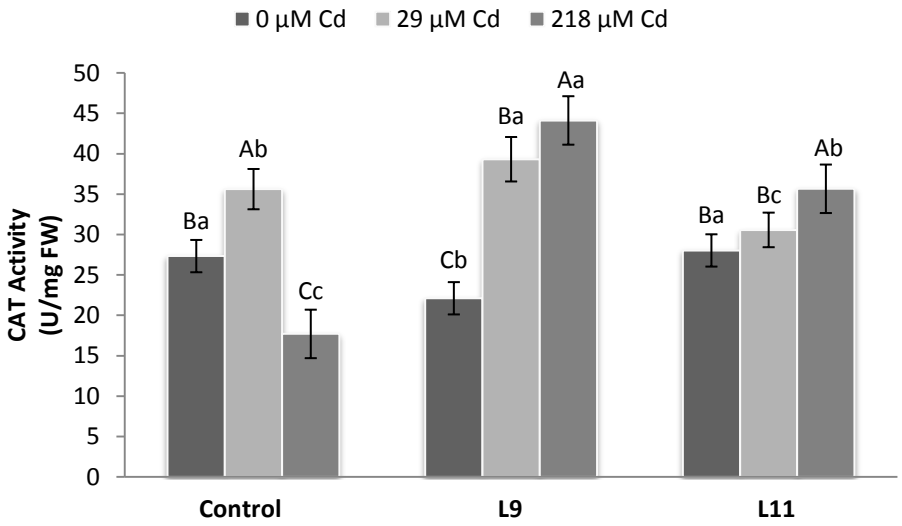


(B)

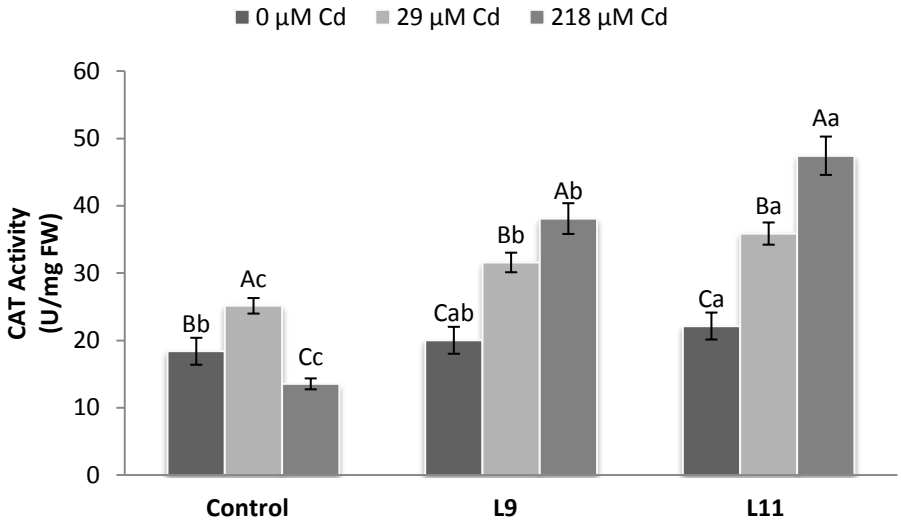
Figure 35. Activity of superoxide dismutase (SOD) in (A) the leaves and (B) roots of control, L9 and L11 plantlelts exposed to 0, 29 and 218 µM Cd for 7 days. Upper and lower case letters are used for comparison among different treatments for the same line, and among different lines for the same treatment, respectively. Means with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$). The vertical bars show standard errors.

5.4.4. Catalase (CAT)

Catalase activity was basically higher in the leaves than roots. In the control treatment (absence of Cd), the leaves of L9 had a lower level of CAT activity than L11 and control leaves (Fig. 36A). On the other hand, there was a higher level of CAT activity in the roots of L11 compared to control and L9 (Fig. 36B). There was no change in the catalase activity in the leaves of L11 in response to addition of 29 μM Cd to the growth media, but the control and L9 exhibited significant increases in the enzyme activity. In contrast, in the roots there was a higher level of CAT activity in line 11 than control and L9. However, L9 still exhibited a significantly higher level of CAT activity than control. Exposure of the plantlets to 218 μM Cd increased CAT activity in the roots and leaves of both lines, while caused significant reduction in those of control plantlets (Fig. 36A&B).



(A)

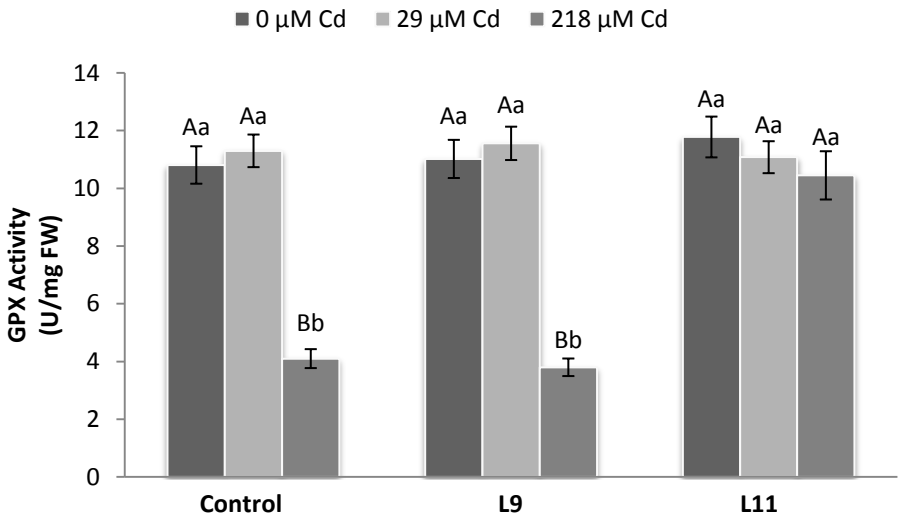


(B)

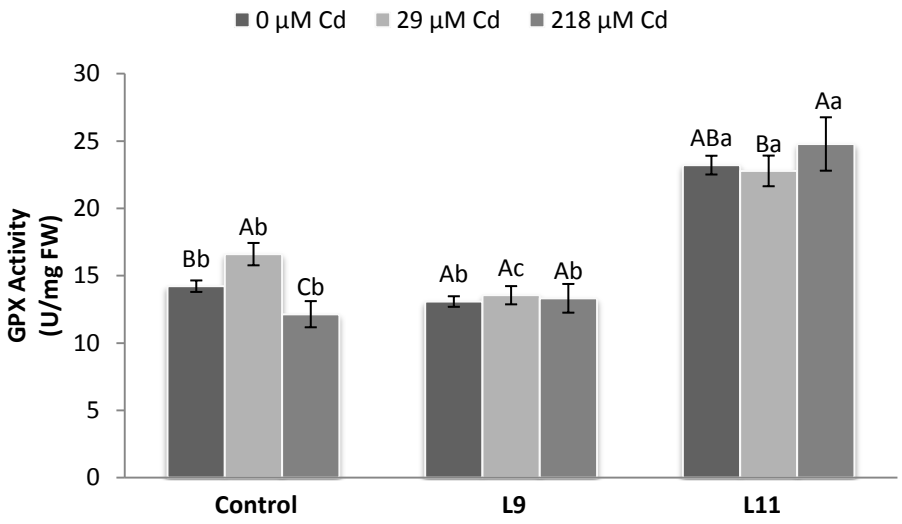
Figure 36. Activity of catalase (CAT) in (A) the leaves and (B) roots of control, L9 and L11 plantlelts exposed to 0, 29 and 218 µM Cd for 7 days. Upper and lower case letters are used for comparison among different treatments for the same line, and among different lines for the same treatment, respectively. Means with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$). The vertical bars show standard errors.

5.4.5. Guaiacol Peroxidase (GPX)

Generally, GPX activity was lower in the leaves than that in the roots (Fig. 37A&B). The same level of GPX activity (~ 11 units) was found in the leaves of the lines and control plantlets when grown in the absence of Cd. The presence of 29 μM Cd in the growth media did not affect GPX in the leaves of all the plantlets compared to when grown in the absence of Cd (Fig. 37A). While exposure to 29 μM Cd did not significantly change GPX activity in the root of the two lines, the enzyme activity was affected in control roots (Fig. 37B). It should be noted that when grown in the absence of Cd, GPX activity in the roots of L11 (23 units) was 10 units higher than that in the roots of L9 (13 units). In response to 218 μM Cd, GPX activity was significantly reduced in the leaves of L9 and control plantlets, whereas it remained unchanged in the leaves of L11 plantlets (Fig. 37A). In contrast, in the roots, GPX activity was not changed in L9, while it was reduced in control but enhanced in L11 (Fig. 37B).



(A)

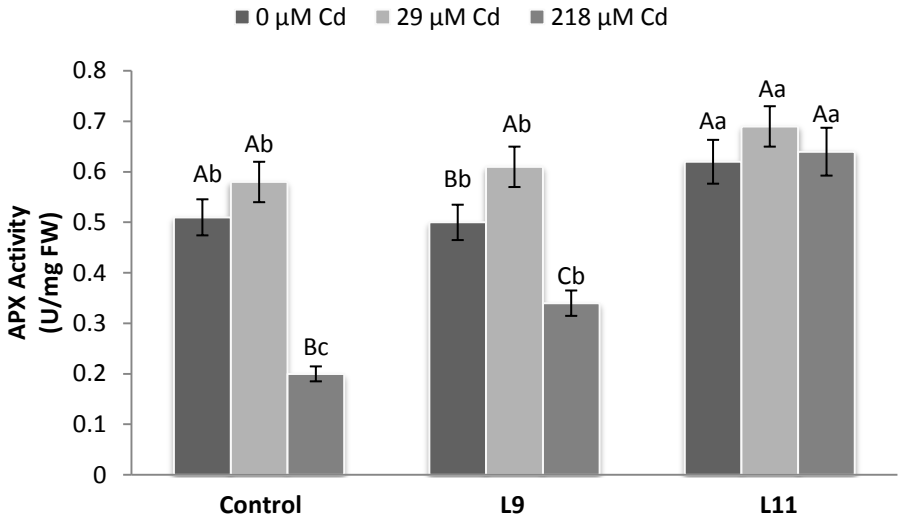


(B)

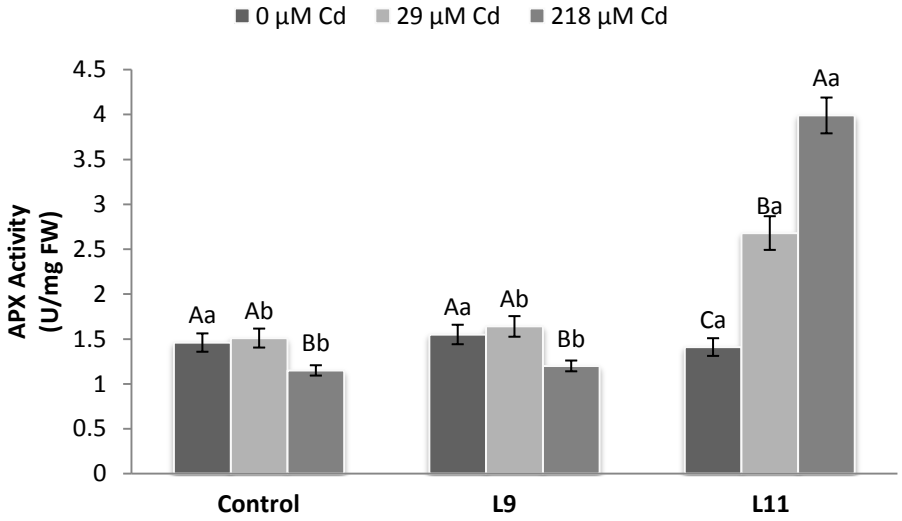
Figure 37. Activity of guaiacol peroxidase (GPX) in (A) the leaves and (B) roots of control, L9 and L11 plantlelts exposed to 0, 29 and 218 µM Cd for 7 days. Upper and lower case letters are used for comparison among different treatments for the same line, and among different lines for the same treatment, respectively. Means with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$). The vertical bars show standard errors.

5.4.6. Ascorbate Peroxidase (APX)

Activity of APX in the roots of control and L9 plantlets was about three times higher than that in their leaves, while this was about double in L11 (Fig. 38A&B). In the absence of Cd, APX activity in the leaves of L11 was higher than that of L9, whereas it was the same in the roots. Supplementation of growth media with 29 μM Cd only brought significant changes to APX activities in leaves of L9 (from 0.5 to 0.6 unit mg^{-1} FW) and roots of L11 (from 1.4 to 2.6 unit mg^{-1} FW) plantlets. The highest Cd concentration (218 μM) used caused an decrease in the APX activity in the leaves and roots of control and L9, while APX activity in L11 remained unchanged in the leaves and was even increased in the roots (Fig. 38A&B).



(A)

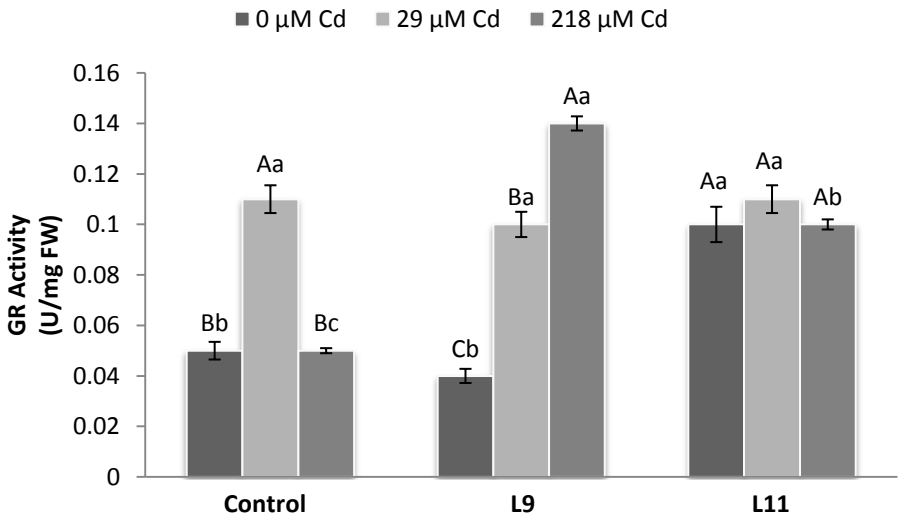


(B)

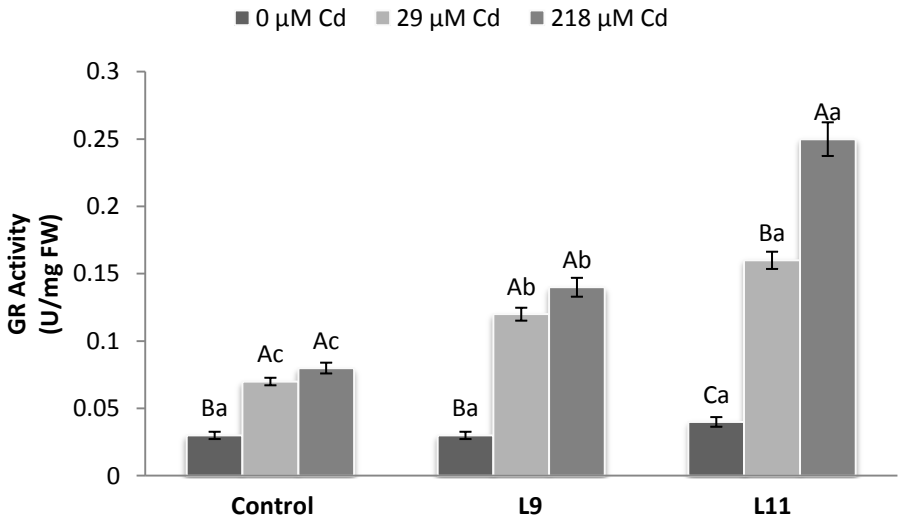
Figure 38. Activity of ascorbate peroxidase (APX) in (A) the leaves and (B) roots of control, L9 and L11 plantlelts exposed to 0, 29 and 218 µM Cd for 7 days. Upper and lower case letters are used for comparison among different treatments for the same line, and among different lines for the same treatment, respectively. Means with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$).The vertical bars show standard errors.

5.4.7. Glutathione Reductase (GR)

In the absence of Cd, GR activity was generally higher in the leaves than roots (Fig. 39A&B). Interestingly, the leaves of L11 plantlets had about 2x GR activity of that in control or L9 (Fig. 39A). When grown in 29 μM Cd-containing media there were significant increases in GR activity in the roots of control as well as L9 and L11 plantlets, while in the leaves GR activity was only increased in control and L9 plantlets (Fig. 39A&B). In the roots, L11 and L9 had significantly higher GR levels (0.16 and 0.12 unit mg^{-1} FW respectively) than control (0.07 unit mg^{-1} FW) (Fig. 39B). This enzyme was not activated in control roots in response to 218 μM Cd and its activity was even decreased in control leaves. On the other hand, GR activity was significantly enhanced in the leaves of L9 and the roots of L11 plantlets (Fig. 39A&B).



(A)

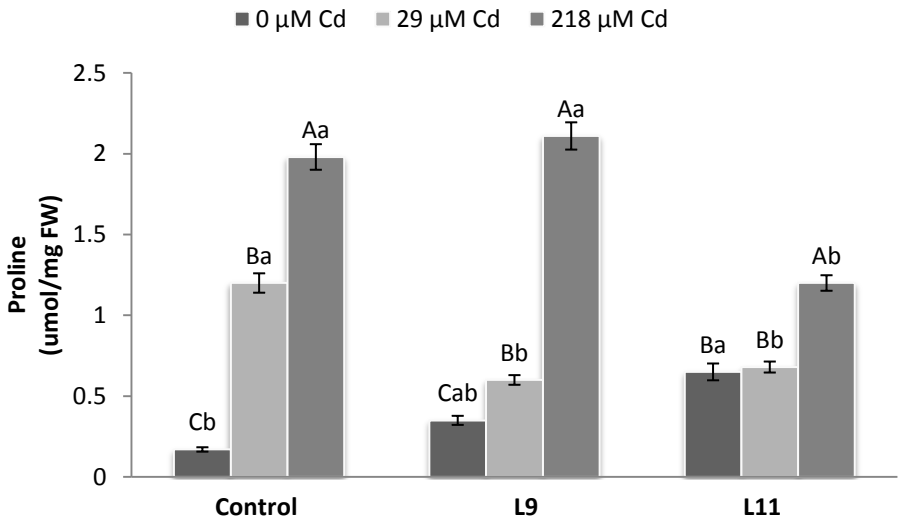


(B)

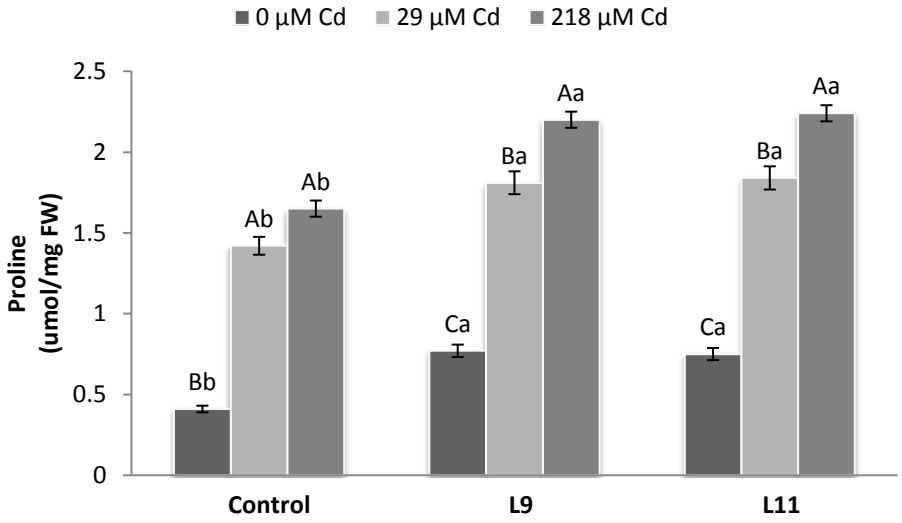
Figure 39. Activity of glutathione reductase (GR) in (A) the leaves and (B) roots of control, L9 and L11 plantlelts exposed to 0, 29 and 218 µM Cd for 7 days. Upper and lower case letters are used for comparison among different treatments for the same line, and among different lines for the same treatment, respectively. Means with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$). The vertical bars show standard errors.

5.4.8. Proline

Except in the leaves of L11 and the roots of control plantlets, proline content in all the other plant materials studied here was increased in a dose-dependent manner with elevated Cd concentrations in the growth media (Fig. 40A&B). In the leaves of L11 plantlets, only treatment with the high Cd concentration (218 μM) caused a significant increase in proline content which was also found in control roots when grown in media supplemented with 29 μM Cd. Control roots contained about three times higher levels of proline than control leaves (Fig. 40A&B). In the absence of Cd, proline content in the roots of L9 and L11 plantlets was higher than that in control roots (Fig. 40B). In the leaves, L11 even contained a higher proline level than L9 (Fig. 40A). In response to growth on media supplemented with 218 μM, proline content was enhanced in both the leaves and roots of L9 and L11, whereas it was only increased in the leaves of control plantlets (Fig. 40A&B).



(A)



(B)

Figure 40. Proline content in (A) the leaves and (B) roots of control, L9 and L11 plantlelts exposed to 0, 29 and 218 µM Cd for 7 days. Upper and lower case letters are used for comparison among different treatments for the same line, and among different lines for the same treatment, respectively. Means with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$). The vertical bars show standard errors.

5.4.9. Chlorophyll

Chlorophyll content in the leaves of L11 was basically higher than that in L9 or control leaves in absence or the presence of Cd (Fig. 41). Growth in the low Cd concentration (29 μM) significantly decreased chlorophyll content (0.5 $\mu g/cm^2$) in control leaves, while that in the leaves of L9 or L11 was not affected. However, treatment with the high Cd concentration (218 μM) decreased chlorophyll content in the leaves of all the plantlets studied (Fig. 41).

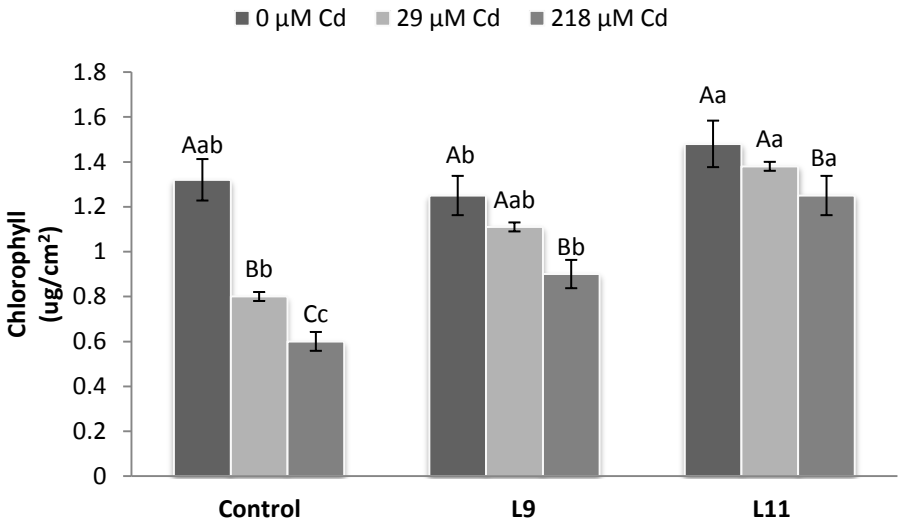
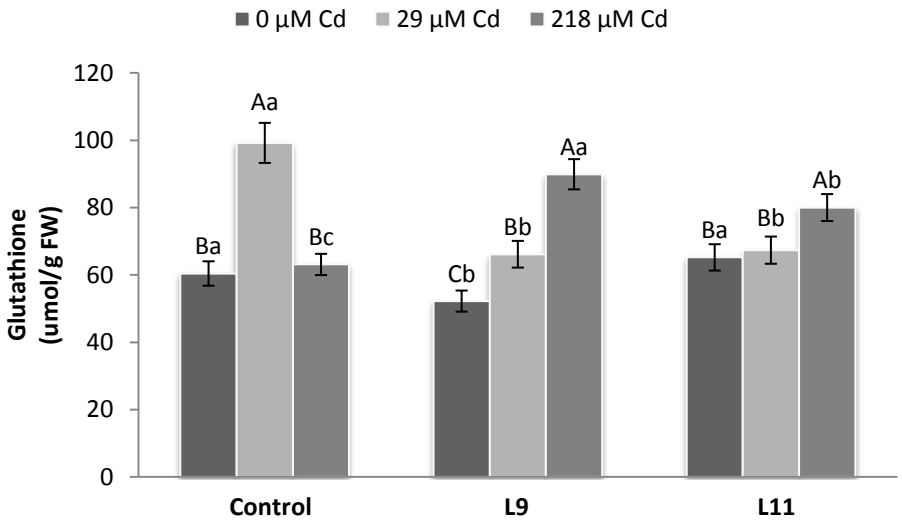


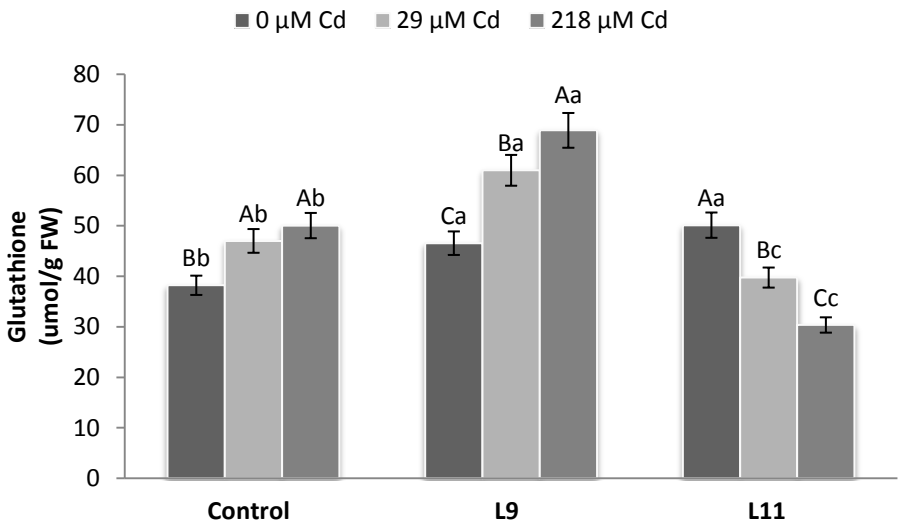
Figure 41. Chlorophyll content in the leaves of control, L9 and L11 plantlelts exposed to 0, 29 and 218 μM Cd for 7 days. Upper and lower case letters are used for comparison among different treatments for the same line, and among different lines for the same treatment, respectively. Means with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$). The vertical bars show standard errors.

5.4.10. Glutathione

After one week of growth in Cd-free media (control treatment), glutathione (GSH) content in the leaves of L9 plantlets was lower than L11 and control leaves (Fig. 42A). Exposure to media containing 29 μM Cd led to increases in GSH levels in the leaves of control and L9 but not in L11. Treatment with 218 μM Cd significantly enhanced glutathione concentrations in the leaves of both the lines whereas decreased GSH content in control leaves (Fig. 42A). On the other hand, control roots contained less GSH than the roots of L9 and L11 (Fig. 42B). When the plantlets of control and L9 were grown in the presence of 29 μM Cd in the growth media, their roots showed increased GSH contents. A further increase in GSH was observed in the roots of L9 exposed to 218 μM Cd (Fig. 42B). In contrast, with the increase in Cd concentration in the growth media, GSH content in the roots of L11 significantly decreased (Fig. 42B).



(A)



(B)

Figure 42. Glutathione content in (A) the leaves and (B) roots of control, L9 and L11 plantlelts exposed to 0, 29 and 218 µM Cd for 7 days. Upper and lower case letters are used for comparison among different treatments for the same line, and among different lines for the same treatment, respectively. Means with the same letter are not significantly different by Duncan's multiple range test ($p<0.05$). The vertical bars show standard errors.

5.4.11. Transmission Electron Microscopy

Electron micrographs showing the ultrastructure of the leaf and root cells of control (mother) plantlets grown in the absence of Cd were obtained to provide a basis for comparison to cells with Cd accumulation in L11 and control plantlets grown in 29 μM Cd-containing medium. A well-structured chloroplast located within a leaf cell of control plantlet grown in the absence of Cd was found (Fig. 43). One plastoglobule at one end of the chloroplast, well-organised grana as well as stroma were seen clearly. In Fig. 44, the structure of a chloroplast in a leaf cell of control plantlets grown in the presence of 29 μM Cd is depicted. In comparsion with the section of leaf cell not exposed to Cd (Fig. 43), some extra dark Cd deposits were located between thylakoids in stroma. The plastoglobule number or size was not affected compared to the treatment in the absence of Cd (Fig. 43). Apart from the chloroplast, some Cd deposits (Figs. 44 & 45) and several lipid bodies (Fig. 46) were also observed in the cytoplasm in the Cd treatment. On the other hand, a choloroplast within a leaf cell of L11 was found to have more and bigger plastoglobuli than in the control and did not accumulate Cd in the stroma as well as cytoplasm (Fig. 47).

There was no Cd particle in the vacuole within a root cell of a control plantlet grown in the absence of Cd (Fig. 48). In contrast, two moderate masses of Cd deposit were found in the vacuole within a root cell of a control plantlet treated with 29 μM Cd (Fig. 49). Moreover, numerous lipid bodies can be seen around the Cd deposits. A vacuole within a root cell of L11 had sequestered a very dense mass of Cd deposit (Fig. 50). There was no sign of lipid body in the vacuole sap.

Cell wall, plasmalemma, middle lamella, and intercellular space in the roots of control (Fig. 52) and L11 (Figs. 53 & 54) exposed to 29 μM Cd were compared to those in a control root grown in Cd-free medium (Fig. 51). In the presence of 29 μM Cd in the growth medium, some Cd particles were sequestered along the cell wall of the control root, but the other structures including plasmalemma, middle lamella and intercellular space were free of Cd. On the other hand, quite intensive Cd particles were sequestered along root cell walls as well as inside the intercellular space of L11. Cd deposits in a high density were also observed at a certain region (unknown) outside the Casparian strip (Fig. 55). This region was observed at a higher magnification (18,000 X) (Fig. 56).

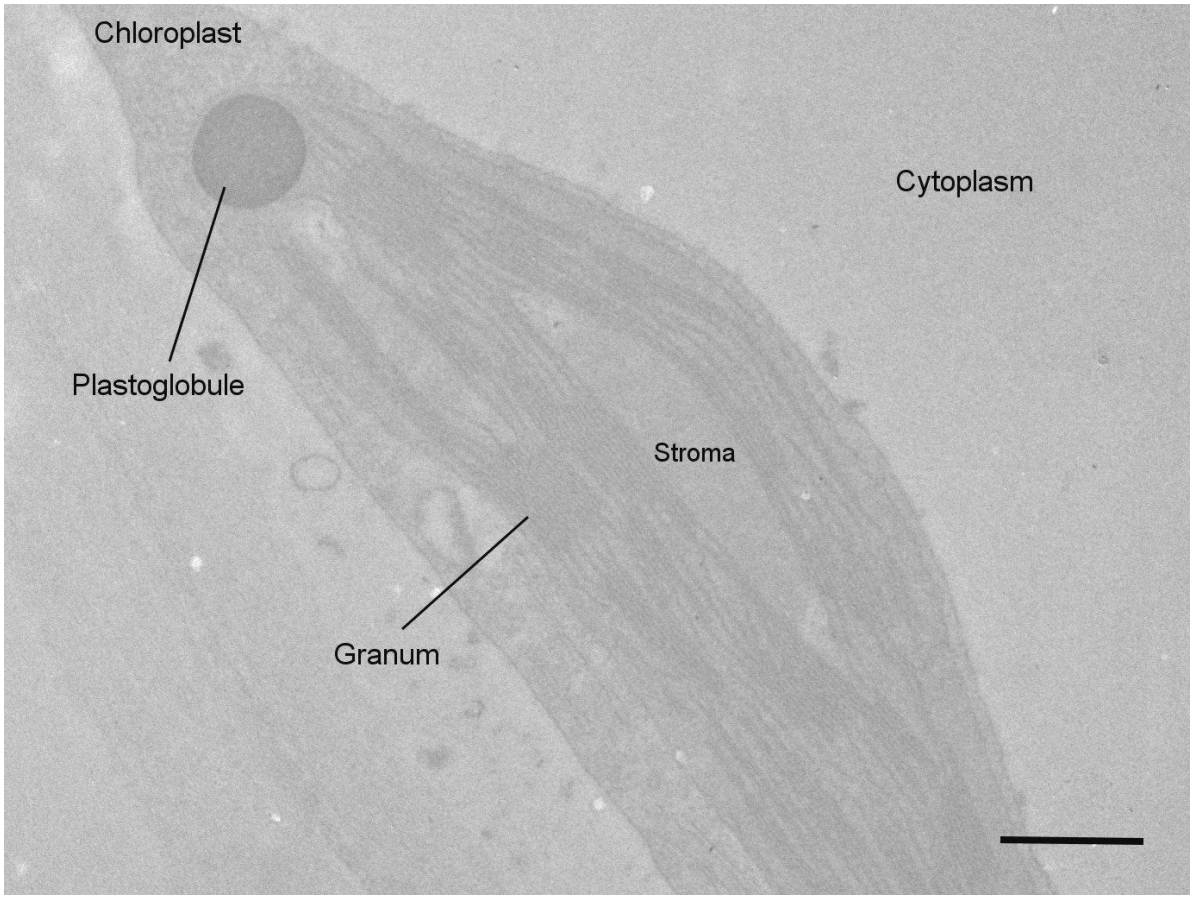


Figure 43. Transmission electron micrograph of an unstained ultra-thin section of a leaf from a 2-week-old control *Solanum tuberosum* cv. Iwa plantlet grown for 7 days in the absence of Cd. The magnification was 22,000 X and the bar=500 nm.

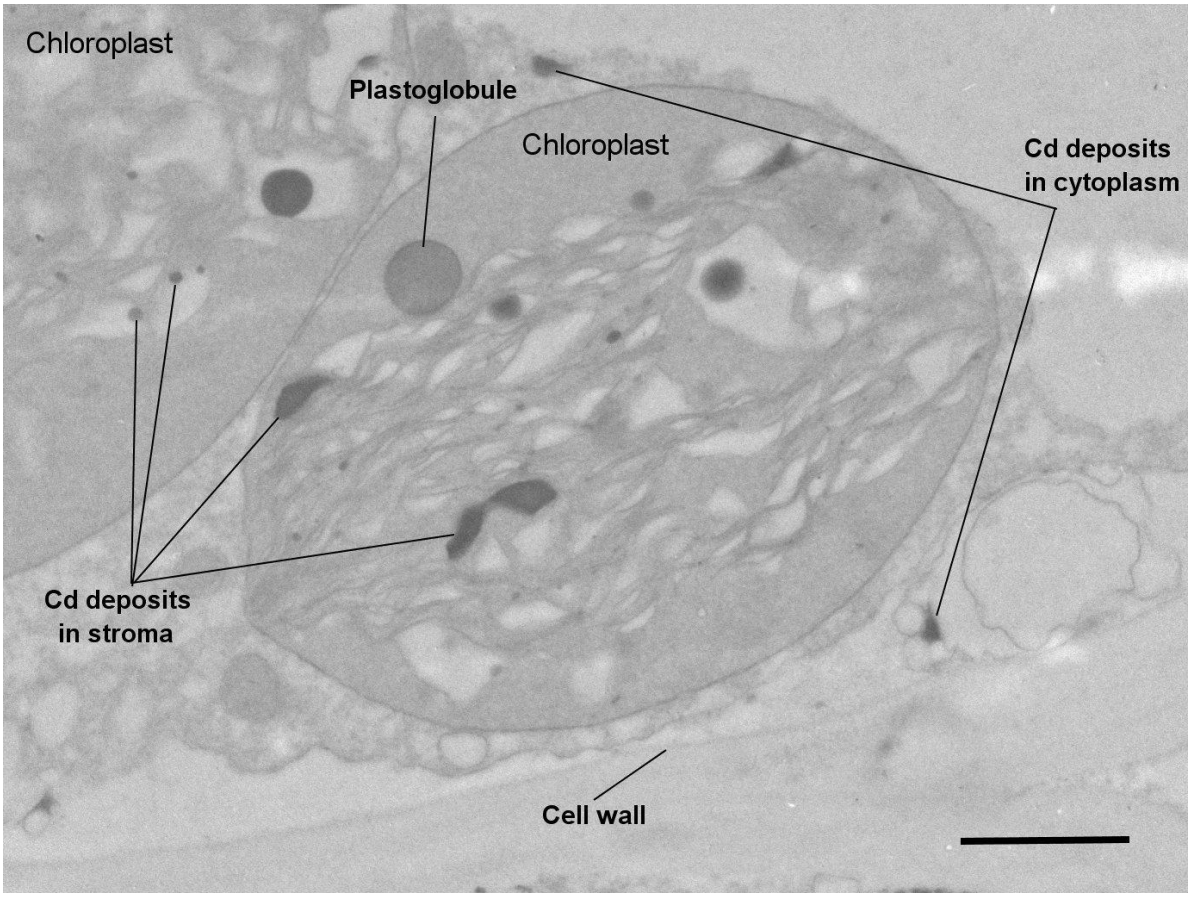


Figure 44. Transmission electron micrograph of an unstained ultra-thin section of a leaf cell from a 2-week-old control *Solanum tuberosum* cv. Iwa plantlet grown in medium containing 29 μM $CdCl_2$ for 7 days. The applied magnification was 11,000 X and the bar=500 nm.

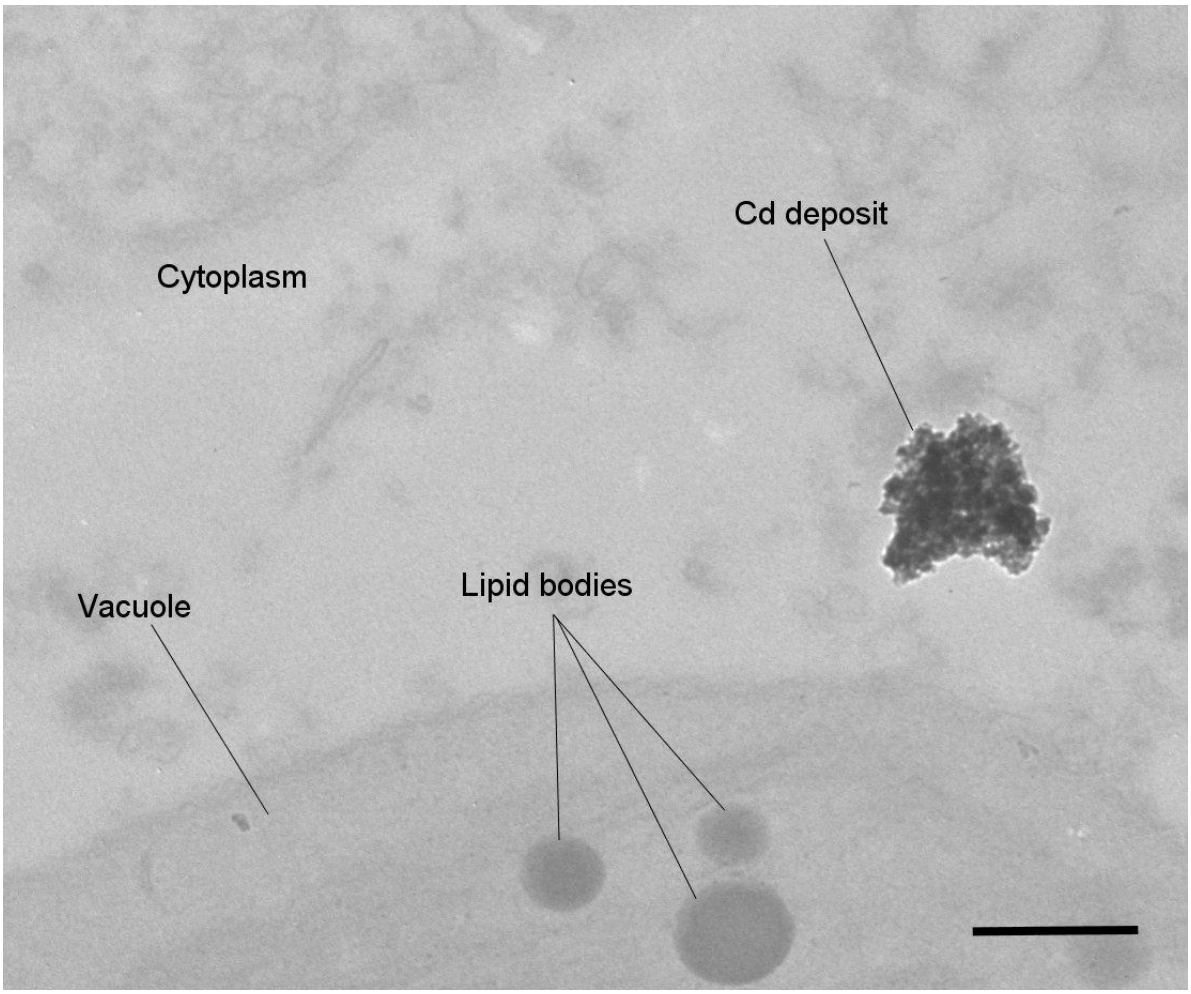


Figure 45. Transmission electron micrograph of an unstained ultra-thin section of a leaf cell from a 2-week-old control *Solanum tuberosum* cv. Iwa plantlet grown in medium containing 29 μM $CdCl_2$ for 7 days. The magnification was 17,000 X and the bar=500 nm.

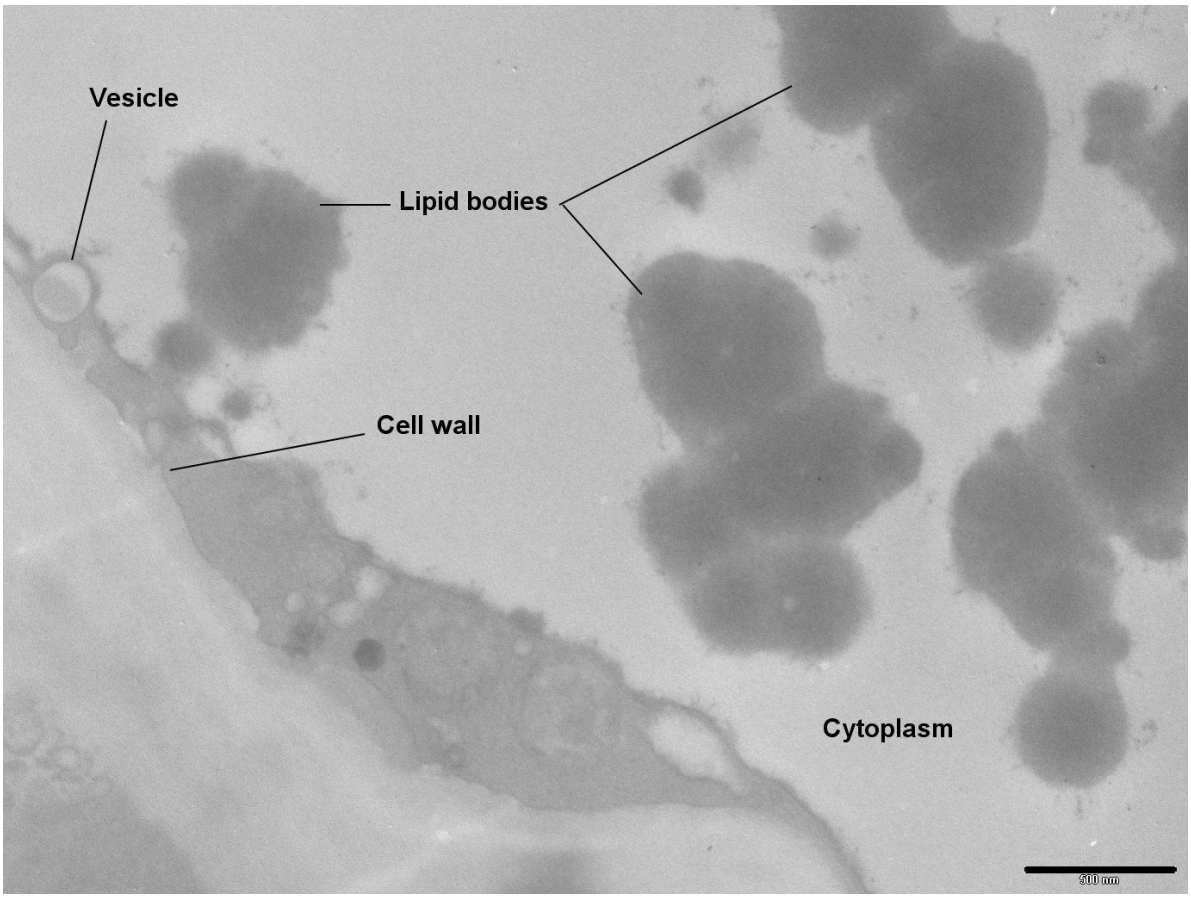


Figure 46. Transmission electron micrograph of an unstained ultra-thin section of a leaf cell from a 2-week-old control *Solanum tuberosum* cv. Iwa plantlet grown in medium containing 29 μM $CdCl_2$ for 7 days. The magnification was 22,000 X and the bar=500 nm.

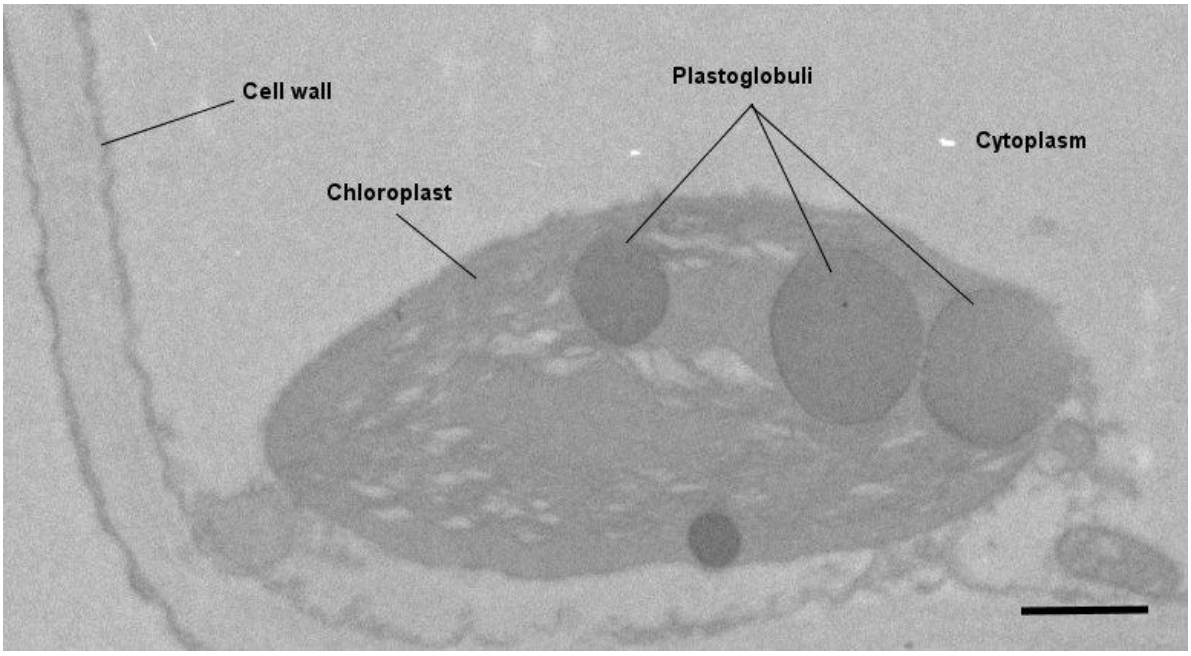


Figure 47. Transmission electron micrograph of an unstained ultra-thin section of a leaf cell from a 2-week-old line11 *Solanum tuberosum* cv. Iwa plantlet grown in medium containing 29 μM $CdCl_2$ for 7 days. The magnification was 7,100 X and the bar=500 nm.

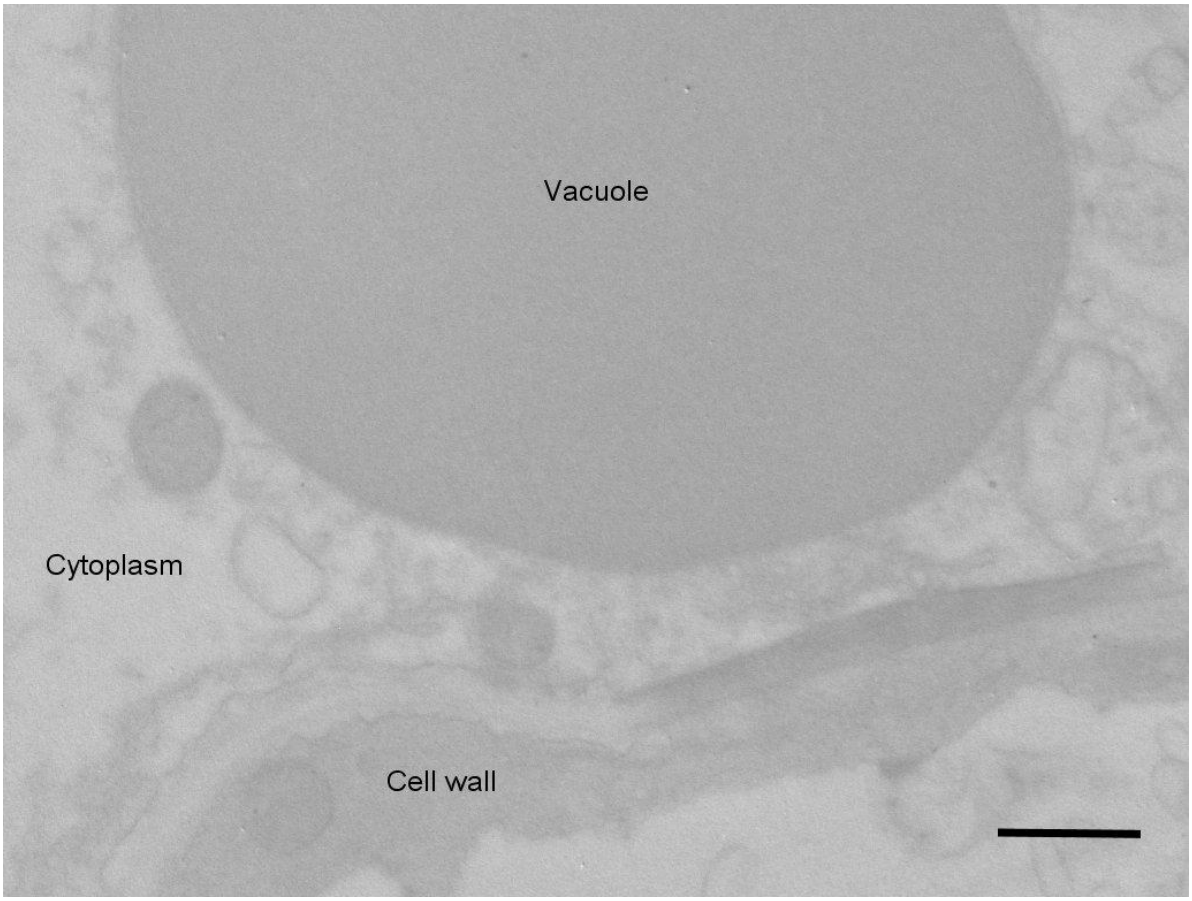


Figure 48. Transmission electron micrograph of an unstained ultra-thin section of a root cell from a 2-week-old control *Solanum tuberosum* cv. Iwa plantlet grown for 7 days in the absence of Cd. The magnification was 20,000 X and the bar=500 nm.

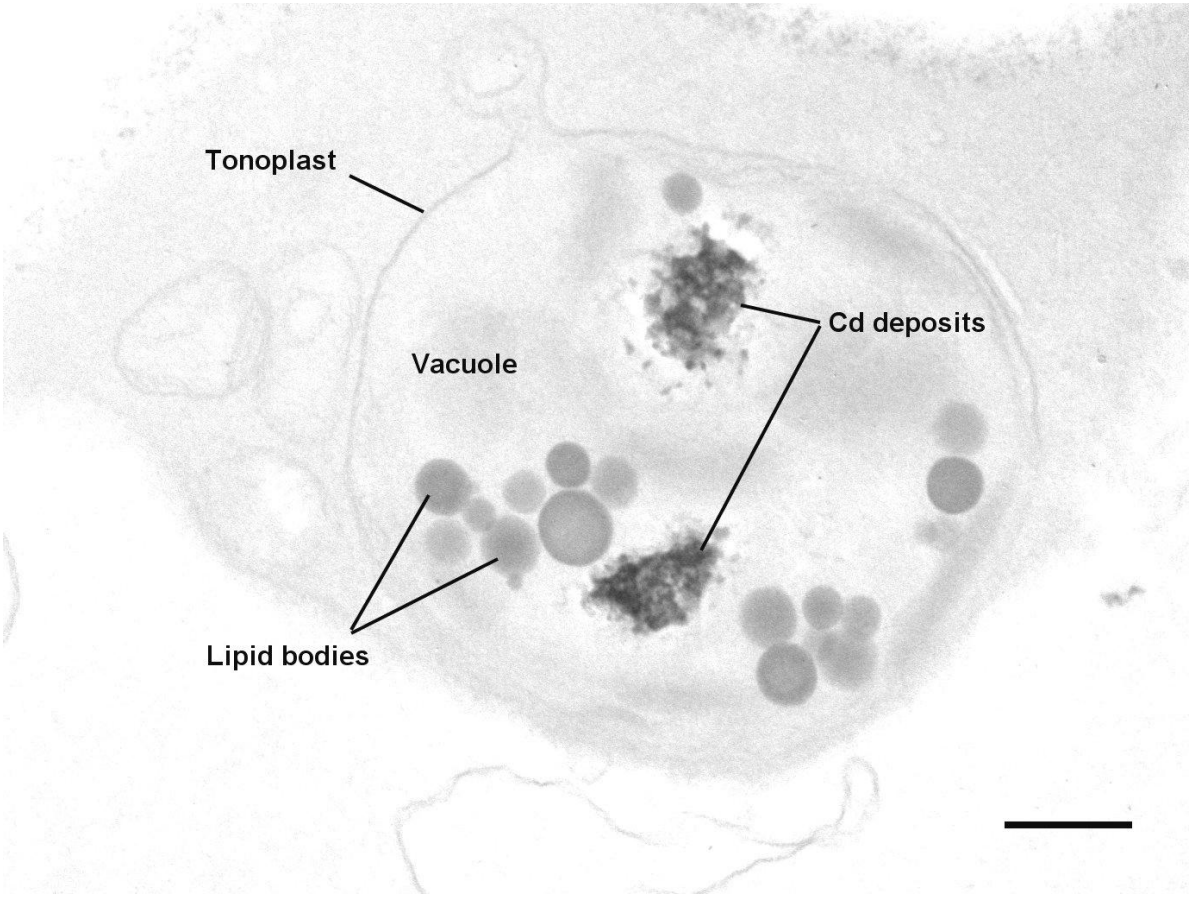


Figure 49. Transmission electron micrograph of an unstained ultra-thin section of a root cell from a 2-week-old control *Solanum tuberosum* cv. Iwa plantlet grown in medium containing 29 μM $CdCl_2$ for 7 days. The magnification was 22,000 X and the bar=500 nm.

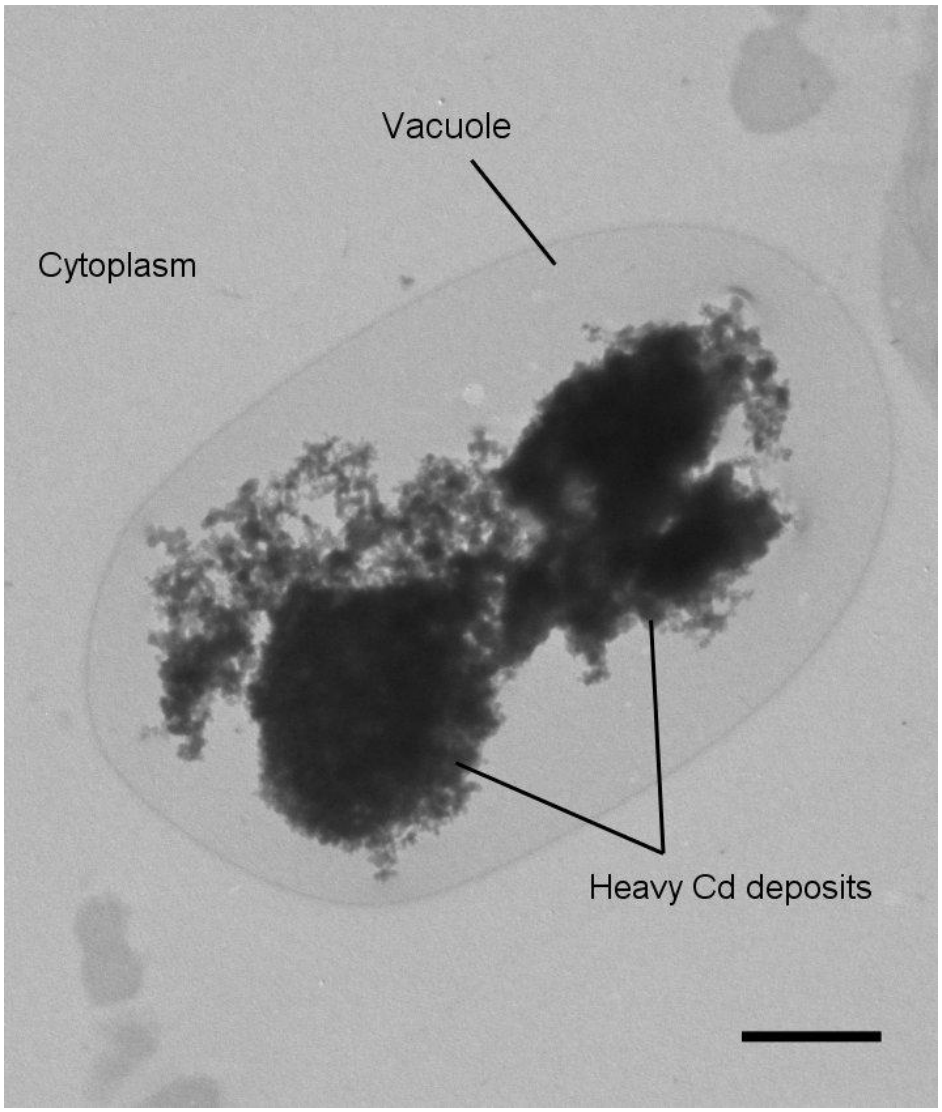


Figure 50. Transmission electron micrograph of an unstained ultra-thin section of a root cell from a 2-week-old line11 *Solanum tuberosum* cv. Iwa plantlet grown in medium containing 29 μM $CdCl_2$ for 7 days. The magnification was 22,000 X and the bar=500 nm.

Figure 51. Transmission electron micrograph of an unstained ultra-thin section of a root cell from a 2-week -old control *Solanum tuberosum* cv. Iwa plantlet grown for 7 days in the the absence of Cd. The magnification was 14,000 X and the bar=250 nm.

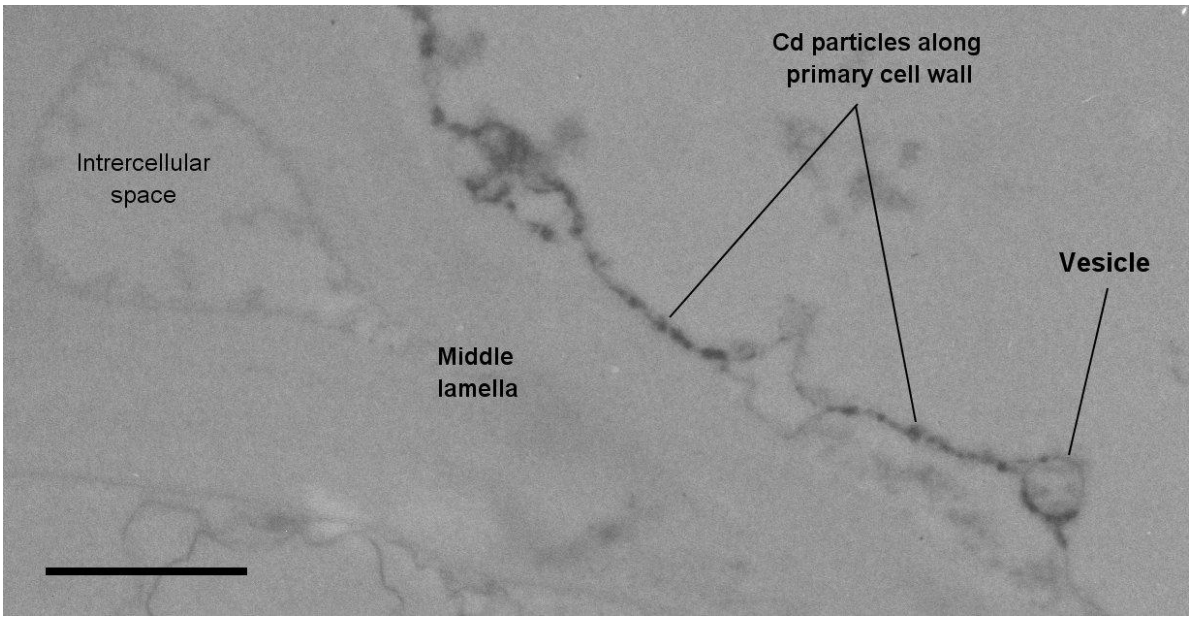


Figure 52. Transmission electron micrograph of an unstained ultra-thin section of a root cell from a 2-week-old control *Solanum tuberosum* cv. Iwa planlet grown in medium containing 29 μM $CdCl_2$ for 7 days. The magnification was 14,000 X and the bar=500nm.

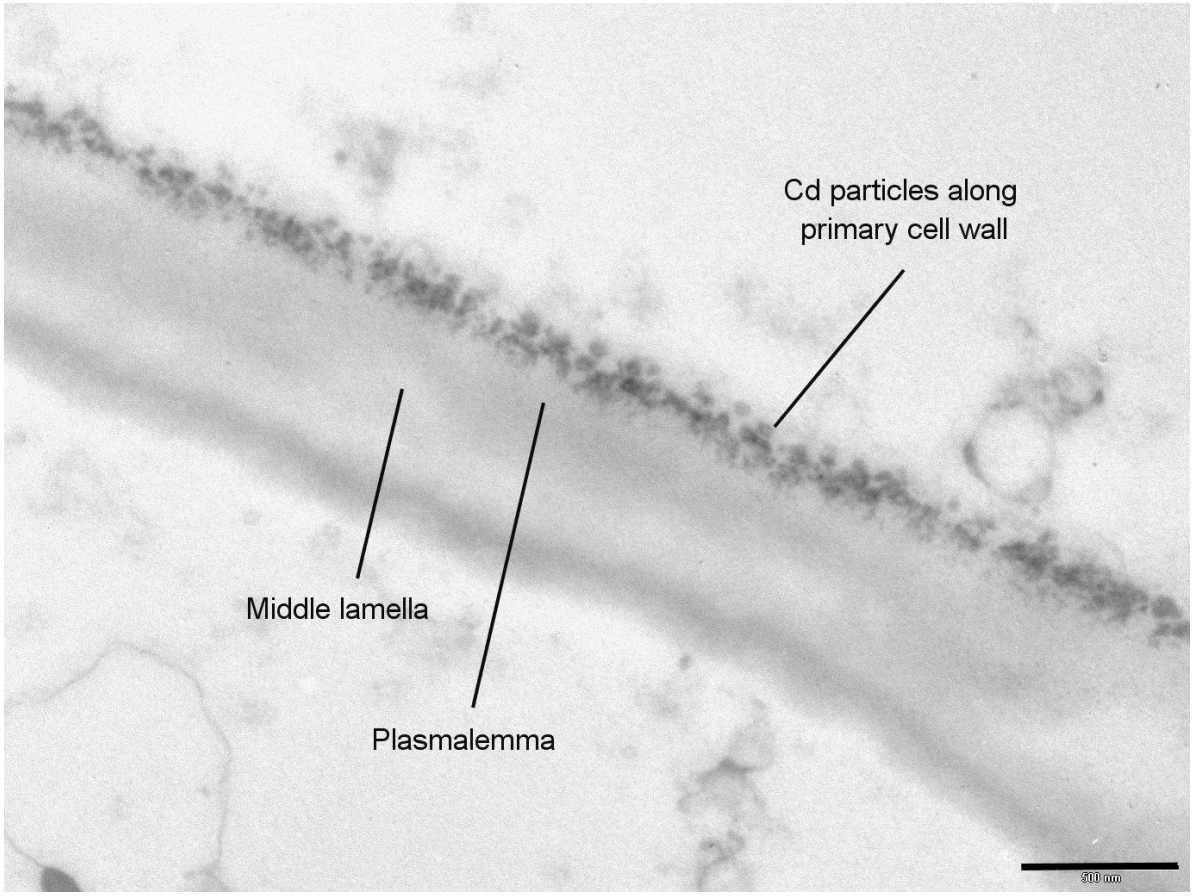


Figure 53. Transmission electron micrograph of an unstained ultra-thin section of a root cell from a 2-week-old line11 *Solanum tuberosum* cv. Iwa planlet grown in medium containing 29 μM $CdCl_2$ for 7 days. The magnification was 22,000 X and the bar=500 nm.

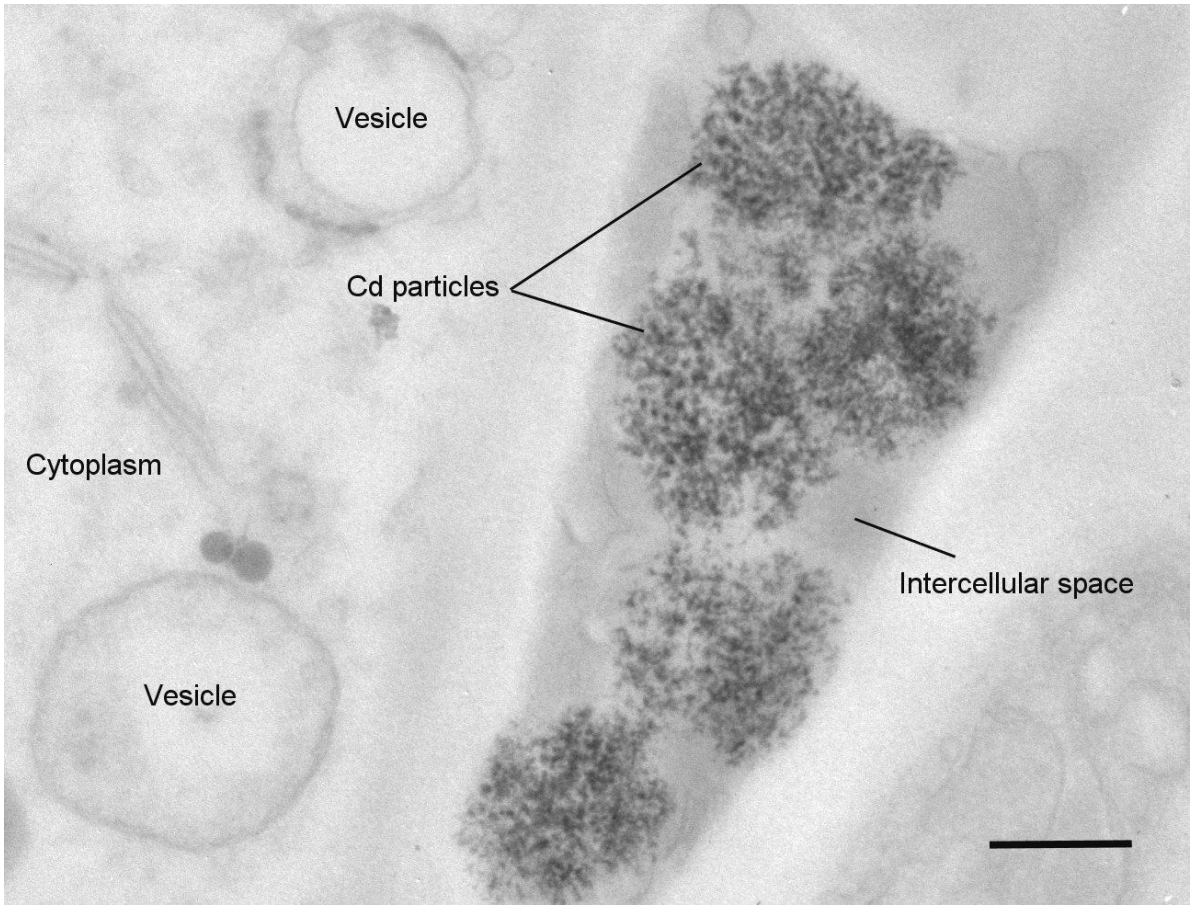


Figure 54. Transmission electron micrograph of an unstained ultra-thin section of a root cell from a 2-week-old line11 *Solanum tuberosum* cv. Iwa planlet grown in medium containing 29 μM $CdCl_2$ for 7 days. The magnification was 28,000 X and the bar=250nm.

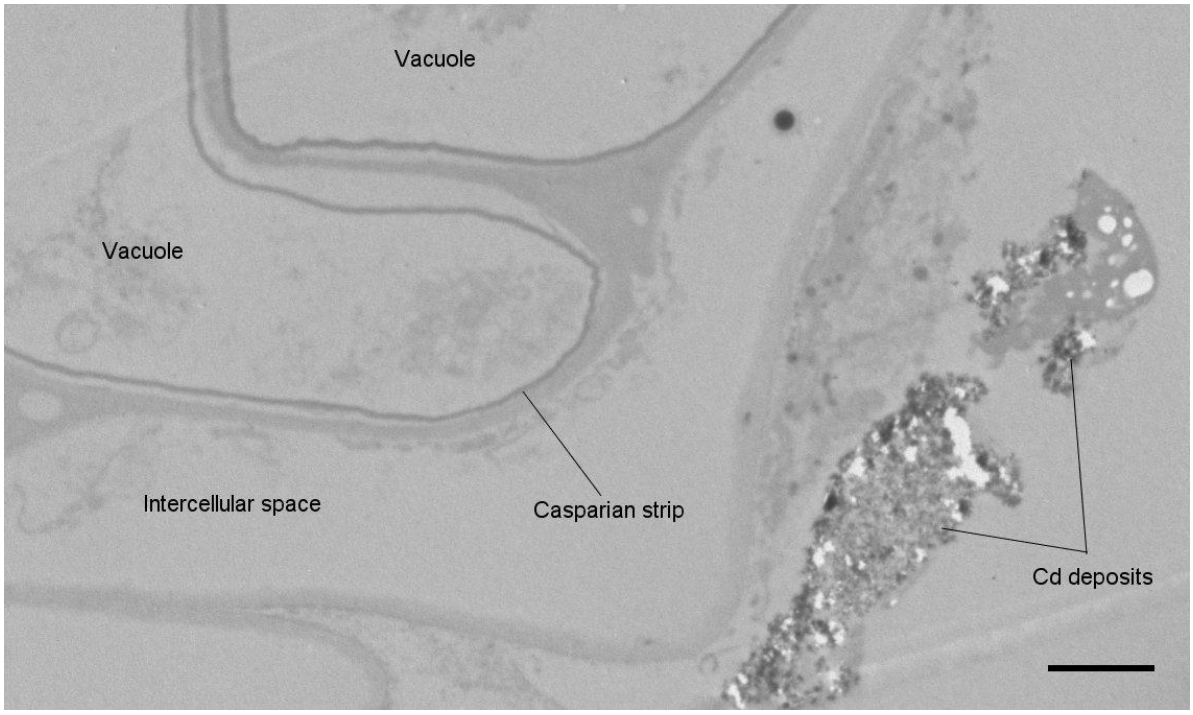


Figure 55. Transmission electron micrograph of an unstained ultra-thin section of a root cell from a 2-week-old line11 *Solanum tuberosum* cv. Iwa planlet grown in medium containing 29 μM $CdCl_2$ for 7 days. The magnification was 4,400 X, and the bar=500 nm.

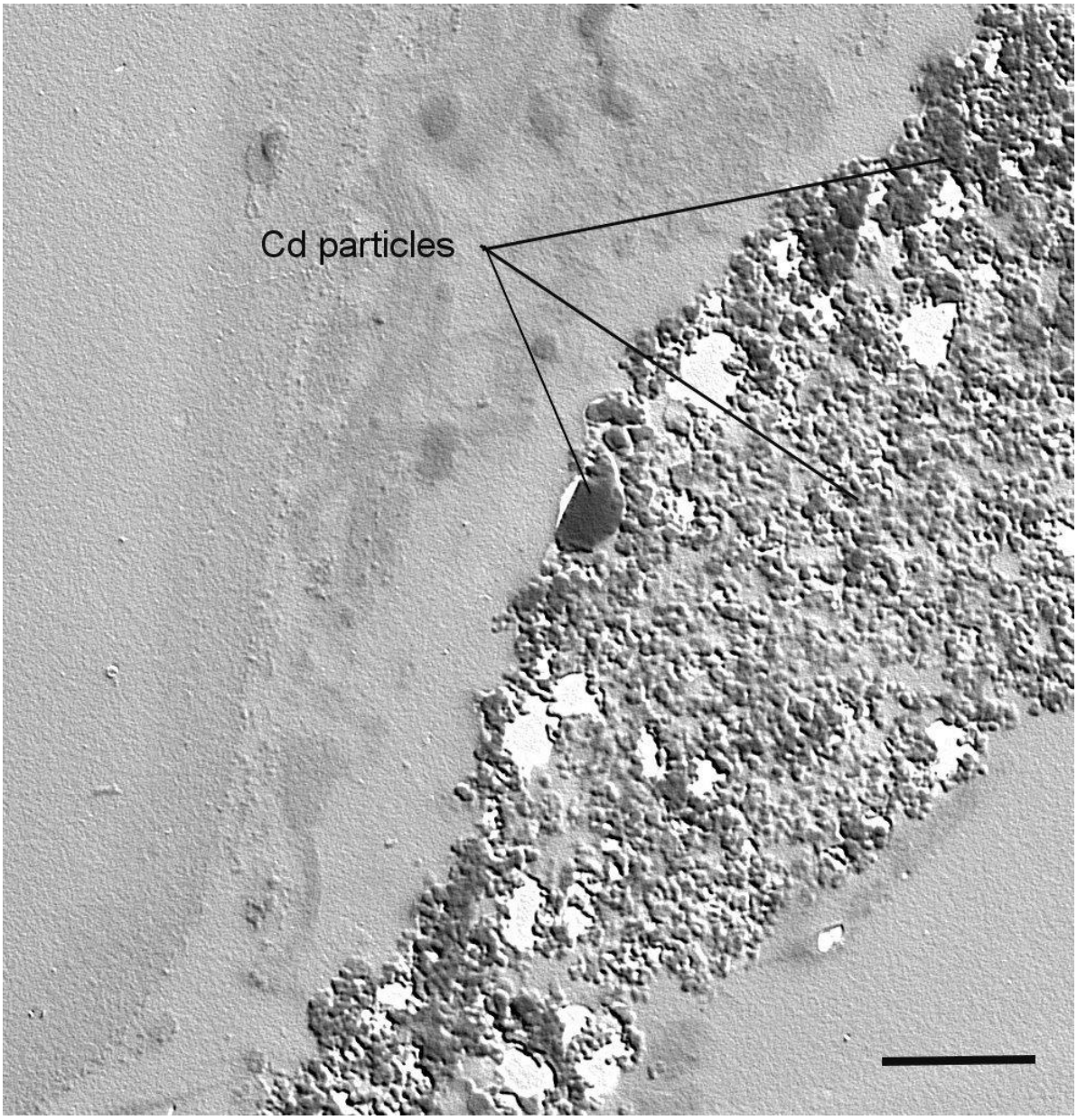


Figure 56. Transmission electron micrograph of the previous section with magnification of 18,000 X. The bar=250 nm. For a better contrast, shading effect was applied using ImageJ software (version-1.48).

5.5. Discussion

The main purpose of this chapter was to obtain some fundamental understanding of the Cd resistance mechanisms that operate in the two new tissue culture derived Cd-resistant plant lines identified based on differential growth abilities and morphological parameters in Chapter 4. Assessing the ROS as well as the respective antioxidative responses in Cd-resistant plant materials is important (Stroiński, 1999). Studying sub-cellular Cd localisation has also been found to be very helpful for a better understanding of Cd resistance mechanisms (Chin, 2007; Phang, 2010). Application of TEM is one of the best methods to investigate Cd compartmentalisation. It can be used to reveal a key Cd tolerance strategy by keeping Cd accumulation away from crucial cell organelles. The root and leaf are the most studied plant parts in these studies due to their crucial roles in Cd uptake and accumulation (Belleghem et al., 2007; Cosio et al., 2006; Vögeli-Lange and Wagner, 1990; Vollenweider et al., 2006). The results of biochemical and microscopic studies showed that morphological differences demonstrated by the new tissue culture-derived Cd resistant potato plant lines (in Chapter 4) were underpinned by their different biochemical and physiological profiles.

Estimation of lipid peroxidation and hydrogen peroxide production was useful in evaluating the extent of Cd-induced oxidative stress in the tissue cuture-derived Cd-resistant potato lines (L9 and L11) compared to that in control plants. In the absence of Cd in the growth media, lipid peroxidation in the roots of both lines was at the same level as that in control roots. Interestingly, hydrogen peroxide level was lower in the roots of L11 and L9 with different levels of significance (11<9<control). In response to Cd exposure, L11 showed the least level of oxidative stress compared to the control and L9.

The results of microscopic studies were considered in conjunction with the biochemical profiles of control and the tissue culture-derived Cd resistant line (L11). Some of the potential strategies minimising Cd-imposed oxidative stress in the roots of L11 were revealed. For example, more Cd binding to the cell wall and compartmentalisation in the vacuoles of the roots of L11 as seen from the TEM micrographs obtained, could have implications for a lower incidence of oxidative damages which would be in agreement with a reduction in membrane lipid peroxidation and hydrogen peroxide production. Fine granular Cd deposits were also evident in the vacuoles of *Arabidopsis* roots exposed to environmentally relevant Cd concentrations (Vangronsveld and Valcke, 2007).

In the roots of L11, the reduced oxidative stress was in agreement with the dose-dependent decrease in the glutathione content which is the direct precursor for phytochelatin (PCs) synthesis and then would be used as the substrate for PC synthesis. Also, this non-protein thiol (GSH) can act as an antioxidant quenching ROS as well (Gill and Tuteja, 2011). Interestingly, in the absence of Cd (control treatment), glutathione level in the roots of L11 as well as L9 was higher than that in control roots. In the literature, there is a positive correlation between Cd accumulation and phytochelatin (PC) content in plant cells (Gill and Tuteja, 2011). This may be related to the key roles of PCs in chelating Cd and mediating transport of Cd complexes into the vacuoles, minimising oxidative stress in the cytosol. Approximately 90 percent of Cd ions in high Cd-resistant tomato cells were found bound to PCs (Goldsbrough, 1991).

Binding of metals to the cell walls is influenced by their ionic and nonionic interactions with pectin, glycoproteins, and other components of the cell walls that could reduce Cd availability, and thus Cd ability to penetrate plasma membrane (Gallego et al., 2012; Hall, 2002). This tolerance mechanism is well-documented in many plant species (Qi-Tang W. U. et al., 2007; Saito et al., 2010; Uraguchi et al., 2009; Wei et al., 2008). This role of cell walls can positively affect tissue cohesion and Cd sequestration as well (Douchiche et al., 2010). Mechanism of cell wall detention was accounted for more than twice the amount of Cd complexation with PC in *Lupinus albus* (Vázquez et al., 2006).

In addition, keeping Cd particles away from cytoplasm by either binding them to root exudates or sequestration of them in intercellular spaces (apoplast) would be the other effective strategies (Hall, 2002). These also appeared to operate in the roots of L11 here. Similar to the finding in this study, Phang (2010) also observed highly dense Pb deposits at a region outside the Casparian strip in *Arabidopsis*. Taking all these together, a higher level of Cd may accumulate in the roots of L11 than its aerial parts which is a typical feature of metal-excluder plants (Sun et al., 2013). This is of great interest as Cd is a potential threat to our health, and then crops with critically controlled Cd accumulation would be of high value. It is suggested that L11 would be a promising material for further exploration along this line of investigations into new food plants (potato in particular) that are much safer to consume (Sun et al., 2013).

Activities of antioxidative enzymes in Cd-tolerant plants are often elevated in response to exposure to Cd (Cuypers et al., 2010). Generally, antioxidative defence is one of the main plant strategies to alleviate the adverse impacts of oxidative stress. Superoxide dismutase (SOD) is known as the first line defender against ROS by scavenging superoxide radicals (Nadgórska-Socha et al., 2013). Catalase (CAT) and ascorbate peroxidase (APX) are the enzymes involved in removal of H_2O_2 produced by dismutation of superoxide radicals or non-enzymatic process in different cellular compartments. The balanced maintenance between ROS and antioxidative system is very critical for survival of plant cells under stress. Noticeably, the present analysis particularly of L11 also confirmed the strong antioxidative defence system in the roots of L11 which was even able to function at the high Cd concentration (218 μM). In contrast, activities of all the enzymes studied in control roots were inhibited. To some extents, the roots of L9 also exhibited a relatively stronger antioxidative defence system compared to control, but this was not as pronounced as the one seen in L11. Thus, the relative differential strengths of Cd resistance seem to be correlated with the antioxidative defence capabilities in coping with potential oxidative damages that could occur in plants under stress.

The roots of L11 showed Cd dose-dependent increase in the activities of antioxidative enzymes, except GPX. This can be explained by the fact that both GPX and CAT enzymes independently catalyse the same reaction (reduction of hydrogen peroxide to water and O_2) (Gill and Tuteja, 2010).

Increase in proline content has been known as one of the plant responses to HM stress (Hayat et al., 2012; Verbruggen and Hermans, 2008). Frequently stress-resistant cultivars have higher levels of free proline (Koca et al., 2007; Madan et al., 1995). In L11, more elevated accumulation of proline compared to control plants could act as a molecular chaperon protecting protein integrity and enhancing the activities of different enzymes (Nadgórska-Socha et al., 2013).

In the TEM micrographs obtained here, the stress factor (cadmium particle deposits) was evident in cytoplasm and stroma (within chloroplasts) of control leaf cells from the treatment with a low Cd concentration. This was accompanied by observation of many lipid bodies in cytoplasm which are also known as stress indicators (Khan et al., 2013). In contrast, particularly the leaf cells of L11 were subjected to a lower level of the stress factor. Therefore, the tissue culture-derived lines, particularly L11, seemed to cope with Cd stress better than control plantlets.

Reduction in chlorophyll content is another Cd stress consequence in plants (Gill and Tuteja, 2010; Khan et al., 2013). For instance, exposure of one-week-old rice seedlings to 50 μM Cd for 15 days resulted in about 25 percent decrease in the leaf chlorophyll contents (Wang et al., 2014a). Therefore, it is expected that Cd-resistant plants can better maintain their chlorophyll content upon exposure to Cd. In the present study, total chlorophyll content in the leaves of both the tissue culture-derived lines (particularly L11) was less affected by the applied Cd treatments compared to control leaves. Sun et al. (2013) compared chlorophyll content in two cabbage varieties of contrasting Cd resistance. The results obtained also showed that chlorophyll content in the resistant cabbage cultivar (Chunfeng) was less reduced than in the sensitive cultivar (Lvfeng).

In the absence of Cd, activities of most of the antoixdative enzymes studied in the leaves of the tissue culture-derived lines were at the same levels as those in control leaves, but L11 showed higher APX and GR activities correlating with its higher Cd tolerance. This was also seen in a Cd-resistant cabbage cultivar (CF) that showed a higher Cd uptake in the roots and lower Cd accumulation in the leaves compared to another cultivar (LF) (Sun et al., 2013).

Activities of most of the enzymes studied were inhibited in control leaves in response to 218 μM, while they were less affected by this Cd level in the leaves of L9. Unlike control leaves, both the tissue culture-derived lines were able to increase leaf proline and glutathione contents in response to 218 μM Cd. However, in response to 29 μM Cd, they were only enhanced in the leaves of L9. These biochemical differences are likely to contribute to the differential responses to Cd exposure exhibited by the different tissue culture-derived lines compared to the control cultured plants.

It can be concluded that both tissue culture-derived lines possess promising characteristics associated with improved Cd resistance compared to the cultured plants used to start the present *in vitro* HM selection research. Of the two lines evaluated here, the plantlets of L11 appeared to also possess Cd exclusion capability to minimise Cd uptake. Due to time restraint, confirmation of this including the use of quantitative Cd uptake measurements must await future studies. However, the findings from the basic physiological, biochemical and ultrastructural observations justify further research on the new plant materials from the present study. They seem to be excellent experimental materials for further research including molecular biology to aid deciphering Cd uptake and resistance mechanisms in potato.

Chapter 6. Summary, Conclusion and Future Directions

6.1. Key objectives, strategies, major findings and limitations

Cadmium accumulation in food crops is a food safety concern (Heppner et al., 2009). Breeding of safer food crops is a desirable endeavour. Genetic variation is a fundamental component of any plant breeding programme (Mohan Jain, 2001). In the present research, naturally occurring (as in genotypes/cultivars already being cultivated) and tissue culture-derived somaclonal variations were explored for their potential implications in genetic improvement of potato germplasm with special emphasis on reduced Cd accumulation (Heppner et al., 2009).

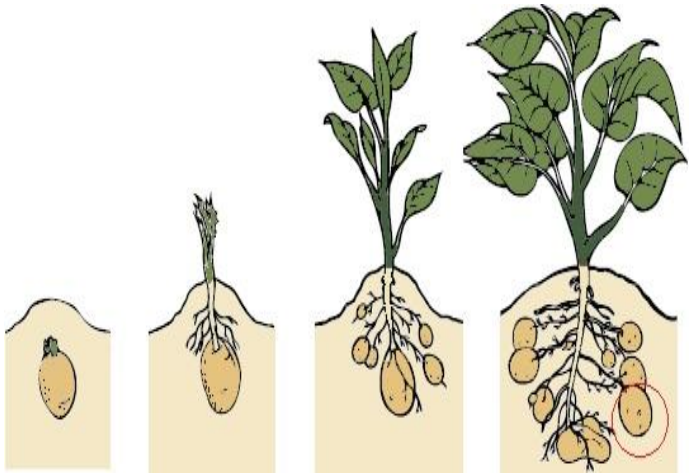
The first approach which took advantages of natural variation occurrence in metal uptake and accumulation among different plant genotypes has been documented (Chen et al., 2012; Hongjiang et al., 2014; Li et al., 2012; Zhan et al., 2013; Zhang et al., 2013; Zhou et al., 2013). There is, however, a paucity of New Zealand-specific information about natural variation in Cd accumulation in tubers harvested from potato varieties grown in typical agricultural soils in regions devoid of traditional pollution-related industries. With this in mind and as a first attempt to start filling in this knowledge gap, a limited and randomly chosen samples of potato varieties were assessed for variation in tuber Cd contents. A major finding was that Summer Delight (SD) showed promise as a low Cd-accumulating cultivar that was high in other desirable mineral contents as well. However, to introduce it as a cadmium safe cultivar (CFC), further studies including evaluation of its Cd accumulation in different growing conditions, particularly high Cd-containing soils are required.

The other way to take advantage of this valuable genotype is improvement of potato germplasm by sexual hybridisation between SD and other potato

genotypes including those high Cd-accumulating cultivars which might be commercially elite varieties or have other desirable attributes. Otherwise, the genes responsible for the low Cd bioaccumulation potential can be identified by molecular studies, and then isolated from SD and transformed to target cultivars (Daghan et al., 2013; Eapen and D'Souza, 2005). Obviously, this will be more complex, costly and subject of the regulations governing genetic engineering of plants in different countries including New Zealand (Du Plessis and Hindmarsh, 2008; Weale, 2010).

On the other hand, three cultivars including Laura (L), Yukon Gold (YG), and Purple Passion (PP) showed a relatively high Cd-accumulating potential. Therefore, it would seem prudent to take more considerations for cultivating them in soils with higher Cd levels. Further studies on the whole potato plants could be really beneficial for understanding mechanisms underpinning Cd uptake from soils and accumulation in potato tubers. Dunbar et al. (2003) found that different patterns of Cd distribution among the potato organs can lead to varietal differences in tuber Cd content. However, this should also be investigated using cultivars grown in the New Zealand agricultural environments.

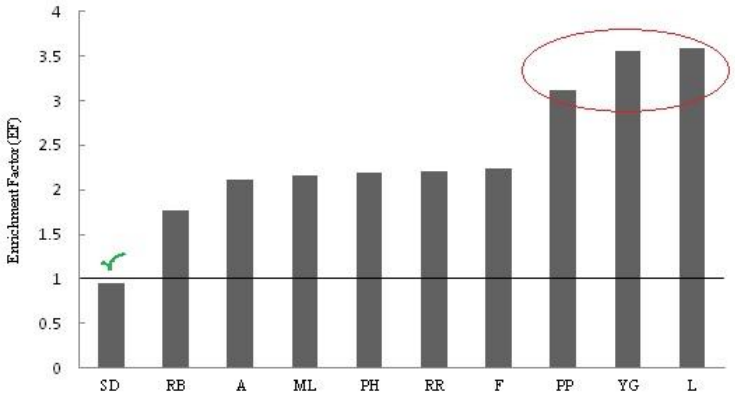
To sum up, in the present research of limited scope within a restricted funding support and timeframe, for the first time the potential variation of different New Zealand potato varieties in taking up Cd and five other elements (Fe, Cu, Zn, Mn, Ca) was evaluated. The procedure of this approach which can be scaled up and yield useful knowledge for consumers and plant breeders is schematically depicted in Fig. 57.



Tuber & Soil Sampling

Sample Preparation for Analysis

Data Analysis



Further Studies

If SD is CSC

Inbreeding

Gene Isolation & Transformation

Germplasm Improvement

Figure 57. Schematic summary of the approach used in this study. The black and red arrows are the taken and future prospective steps, respectively.

Exploitation of somaclonal variation in the second approach was successful in identification of a tissue-culture-derived potato line (herewith called L11) with improved Cd resistance. L11 showed high Cd tolerance as well as potential of Cd exclusion from uptake, making it of high interest for minimising potato Cd level. Towards this outcome, an important key was the investigation into the two types of calli (A and B) exhibiting distinct morphologies that were induced from the same initial plant materials. More importantly they exhibited differential Cd sensitivity which had not been realised in potato callus research before. At this stage of working on potato callus cultures, unexpectedly a novel phenomenon of microtuberisation from callus (type-B) cells was revealed for the first time in the world (Ashrafzadeh and Leung, 2015). This should be useful for basic research about microtuber induction. Also potato industry in the future could develop this protocol as a potential way for microtuber production in a very minimal space requirement and time frame.

In the 18 Cd treatments for the *in vitro* selection of calli, two parameters including the impacts of exposure duration (1-5 months) and exposure start time (from callus initiation or proliferation), that were neglected in previous *in vitro* selection studies, were investigated. As both Cd resistant lines (L9 and L11) were derived from calli initiated on Cd-containing media, it can be suggested that early Cd exposure may increase the chance of somaclonal variation occurrence. On the other hand, it seemed that development of desirable somaclonal variants is not necessarily correlated with the prolonged Cd exposure periods. Indeed, none of the lines derived from calli exposed to Cd in the callus proliferation medium for more than 4 months showed notable Cd resistance. Therefore, exposure timing may be more crucial for generation of somaclonal variation than exposure duration in this case of *in vitro* selection of Cd resistance variation. This has not been found before.

An important aspect of *in vitro* breeding programme was the need to minimise the number of transient variants (carrying epigenetic changes) to the *in vitro* screening stage. Here, sub-culturing all the selected lines in Cd-free media for at least three months was found to be effective for this objective. Furthermore, the experimental design for *in vitro* screening of the tissue culture-selected lines under two different Cd levels (low and high, respectively) was thought to be important. The results clearly confirmed this although three of the five lines screened were more resistant compared to the control plantlets at the lower Cd level, they could not perform as well as L9 and L11 in response to the higher Cd level.

L11 was only generated after regeneration from Cd-selected type B potato callus in the present research. Therefore, it is a novel Cd resistant plant material and offers a unique opportunity for studies into the basic mechanisms of Cd resistance. L11 plantlets may exhibit superior Cd resistance than the control plantlets but it remains to be confirmed if it has a lower Cd bioaccumulation potential. Given the time constraints of this project, priority considerations were given to first carrying out morphological characterisation of the tissue culture-derived Cd resistant plants, biochemical analysis regarding the status of ROS and antioxidative enzyme activities and ultrastructural characterisaion of the cells of the resistant and control plants to find clues if and where Cd might be accumulated in their cells. This approach is schematically depicted in Figure 58.

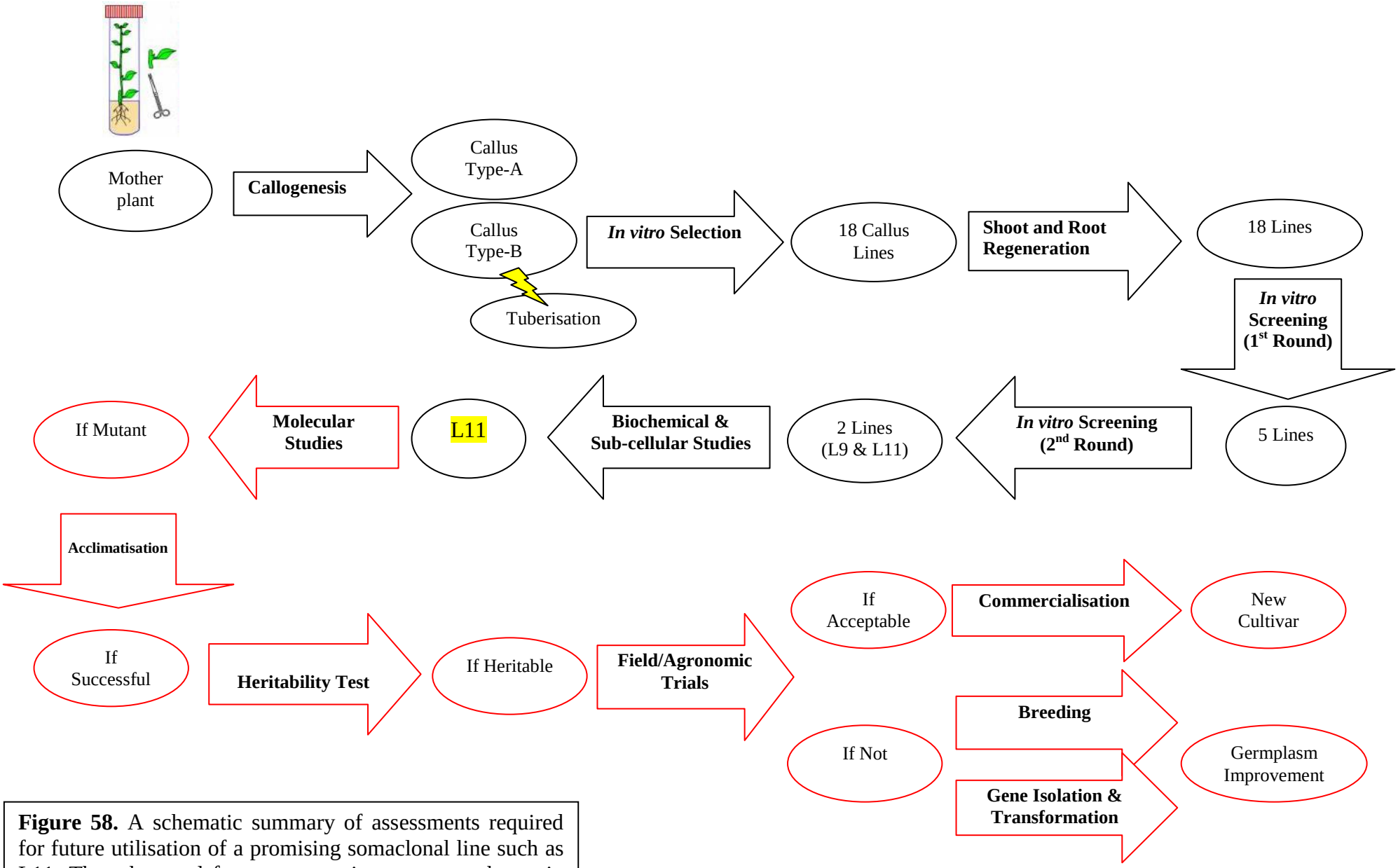


Figure 58. A schematic summary of assessments required for future utilisation of a promising somaclonal line such as L11. The taken and future prospective steps are shown in the black and red arrows, respectively.

6.2. Cd resistance mechanisms

The results of biochemical and microscopic studies suggested some of the key characteristics of L11 including reduced ROS generation, enhanced abilities in antioxidative defence, possible capability to limit Cd uptake into root cells, Cd binding to the cell wall, and Cd sequestration in the intercellular spaces and vacuoles compared to the control plants. However, more time beyond the present PhD study was needed to determine the exact Cd resistance mechanism underpinning the strong plant growth performance of L11 in response to Cd. Therefore, further deeper characterisations of this new Cd resistant plant line are highly recommended before it can be assessed by the agricultural industry as a potentially new breeding line or cultivar for use. Molecular genetic techniques involving application of molecular markers such as RAPDs, RFLPs, AFLPs and SSRs (Mohan Jain, 2001) should be employed to probe the variation in response to Cd exhibited by L11 (and Summer Delight) at the molecular level. The heritability of the Cd resistance traits of L11 should be investigated as well.

The Cd resistance mechanisms exhibited by L11 may be associated with a broad range of alternations in nuclear and cytoplasmic genetic elements (Lestari and Endang Gati, 2006; Rai et al., 2011). Some examples of genetic changes leading to the elevated Cd resistance capability of L11 may include point mutations, DNA methylation and alternation in copy number, chromosomal rearrangements and recombination, and may even involve transposable elements (Lestari and Endang Gati, 2006). It is well-known that the chance of the occurrence of these changes are higher in polyploid species such as potato (Mohan Jain, 2001).

Obviously, some of these genetic changes could be reflected in alterations in gene expression in L11. The changed profile in proteins can either have regulatory roles in Cd resistance mechanisms or may be directly involved in metal uptake or transfer process. For instance, transition metal transporters are proteins facilitating uptake of metal ions from soils, transporting them to plant organs, and compartmentalising them within cells. As it is shown in Fig. 59, all Cd transporter proteins identified so far are associated with either tonoplasts or plasma membranes. No transporter has been found for Cd transfer into the plastids. In contrast, in the present study, the Cd deposits were seen in the chloroplasts of control leaf cells. It means that there should be a transporter for Cd transfer across chloroplast membranes at least in potato. Alternatively, ZIP4 may facilitate Cd transfer as well as Zn. However, this is only a hypothesis as this transporter has not been even found in the leaf yet [Table 18; (Hall and Williams, 2003)].

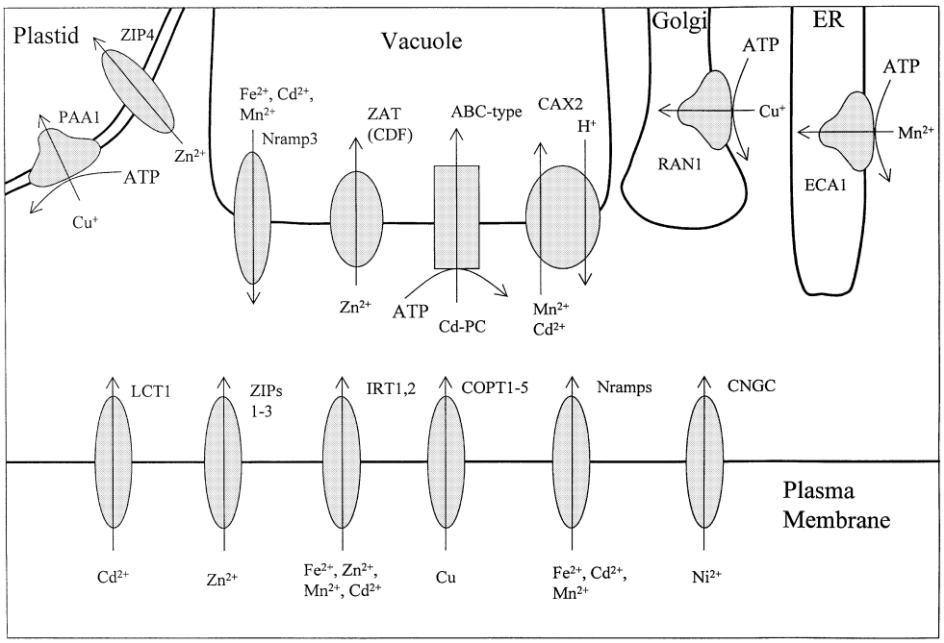


Figure 59. Putative transition metal transporters in plants (Hall and Williams, 2003)

Table 18. Characterisation of identified transition metal transporters (Hall and Williams, 2003)

Name	Family members in *A. thaliana*	Proposed specificity	Cellular location	Main tissue expression
Heavy metal ATPases (P_{1B})	8			
RANI		Cu	Post-Golgi	Whole plant
PAA1		Cu	Chloroplast	
Ca^{2+}-ATPases (P_{2A})	4			
ECA1		Mn^{2+}	ER	Roots?
Nramp	6 (+EIN2)			
AtNramp 1		Fe, Mn		Roots
AtNramp 2		–		Roots
AtNramp 3		Fe, Cd, Mn	Vacuole	Roots/shoots
AtNramp 4		Fe, Mn		Roots/shoots
OsNramp 1		Mn, Fe		Roots
OsNramp 2		Mn		Leaves
OsNramp 3		Mn		Roots/leaves
LeNramp1		Fe		Root vascular parenchyma
CDF	8			
ZAT (AtMTP1)		Zn	Vesicular/vacuolar	All tissues
TcZTP1		Zn	Intracellular membranes	Leaves, roots
TgMIP1		Cd, Co, Zn, Ni	Vacuole	Leaves
ShMTP1		Mn	Intracellular membranes	Roots, leaves
ZIP	15			
IRT1		Fe, Zn, Mn, Cd	PM	Roots
IRT2		Fe, Zn		Roots
OsIRT1		Fe		Roots
LeIRT1		Fe (broad?)		Roots
LeIRT2		broad?		Roots
TcZNT1		Zn, Cd		Roots, shoots
TcZNT2		–		Roots
ZIPs1-3		Zn		Roots
ZIP4		Zn	Plastids	Roots, shoots
GmZIP1		Zn	Peribacteroid membrane	Root nodules
Cation/H^+ antiporters	12			
CAX 2		Ca, Mn, Cd	Vacuole	Roots
ABC transporters	128	PC-Cd, GS-Cd		
ID17		Fe		
COPT1-5	5	Cu		Leaves, stems, flowers
GNGC channels	20	Ni, Pb	PM	

As it is shown in Table 18, there is no transporter specific only for transfer of Cd, and all Cd transporters can also facilitate the transfer of some other metals which are micronutrients essential for plant growth including Fe, Zn and Mn. Hence, any changes in number or activities of these transporters may affect the transport of all the respective metals together. For crop species, this should be a critical consideration, as crops are our main sources of micronutrient intake (Welch and Graham, 2004). It is, therefore, of great interest in future to fully characterise the metal transporter profiles and activities in L11 as well as the micronutrient contents compared to control plants.

Quantifying the expression of genes encoding these transporter proteins, facilitating Cd transfer into either the cytoplasm (IRT1, NRAMP) or the vacuole (ABC-type) would be of great interest (Brunetti et al., 2015; Hartke et al., 2013). This is currently possible by using new techniques such as quantitative reverse transcription PCR (RT-qPCR) in potato which its genome is fully sequenced. Evaluation of expression level of phytochelatin synthase gene (*PCS*) can be exploited as the other Cd resistance biomarker due to the key roles of phytochelatin in Cd detoxification and sequestration (into the vacuole) processes (Stroiński et al., 2010).

Another Cd resistance mechanism observed in L11 was the enhanced ability of the cell wall of the root in Cd binding. It should be investigated whether the cell walls possess different binding affinities to different metals or not. It is only known that increase in polysaccharides of cell walls is correlated with metal binding but how this can regulate binding of different metals remains to be investigated further.

6.3. Non-GMO status of L11 and future prospects

The public should be informed that a new plant material such as L11 or Summer Delight, for example, was originally sourced from tissue culture selection or natural variation, respectively, is not derived from genetic engineering procedures and therefore it is not considered as a GMO (genetically modified organism). The practical application of L11 would also be greatly facilitated if this new material has the nutritional value in addition to a sought-after trait such as improved Cd resistance, particularly related to low Cd bioaccumulation (Rai et al., 2011).

If L11 is validated to be a new breeding material of low Cd accumulation potential, it is necessary to evaluate their performance in the field. To be of use for future cropping, it should have acceptable agronomic attributes in different environments in the field. For this aim, acclimatisation technique can help *in vitro* culture derived plants to become adapted to the natural environment gradually. However, besides introducing L11 as a new cultivar to the public, like Summer Delight, it can be alternatively hybridised with other cultivars to breed new potato cultivars with low Cd accumulation in a range of potato germplasm. Moreover, it can be used for identification and then isolation of the respective genes (which are responsible for Cd resistance or regulating Cd bioacccumulation) for transformation to other potato genotypes. Through this approach, Cd resistance was obtained in *Staphylococcus lugdunesis* by cloning and expression of a Cd resistance gene, *cadD* from *S. aureus* (Crupper et al., 1999). Both of these approaches can lead to improvement of Cd resistance and Cd bioaccumulation in potato germplasm. In future, a comparative study of both SD and L11 should also be of interest in relation to their response to growth media containing Cd and their Cd accumulation potential.

All in all, against the global gradual increase in the average level of human Cd intake (Satarug et al., 2010), consumption of crop cultivars with a low Cd level will be a practical strategy. Towards this end based on the possibility of using genetic improvement of crop plants, in the present PhD research two approaches were applied on the selected food plant potato which could account for more than 50 percent of our Cd intake (Dunbar et al., 2003). The first approach (screening of New Zealand potato cultivars for variation in Cd-accumulating ability) has led to identification of one low-Cd cultivar already grown commercially in New Zealand, and the second approach (*in vitro* breeding of Cd-resistant variants) has resulted in development from a model experimental potato variety Iwa of one new potential cultivar of increased Cd resistance and possibly of low Cd-accumulating potential. Therefore, genetic improvement of potato germplasm for low Cd bioaccumulation seems to hold promise to contribute to the quest for better food safety worldwide. To characterise this plant tissue culture-derived line L11, so far only a few studies were carried out due to the time limitation of this thesis. Obviously, further characterisations of this line will be more beneficial. The studies would require L11 and control culture plants acclimatised and grown to maturity and tuberisation under glasshouse and field (*in vivo*) conditions. Therefore, at present, the results obtained from biochemical and microscopic studies do not enable drawing a comprehensive picture of Cd resistance mechanisms of L11. One of the reasons is complexity of Cd accumulation process in the potato. Since bioavailable Cd ions in the soils for loading into the potato tubers are required to take a long journey after Cd uptake by the roots: the roots to shoots, shoots to leaves through the xylem, and then back down into the tubers by the phloem (Reid et al., 2003). Hence, for practical application of this potential Cd-resistant cultivar, all the questions raised and discussed here need to await future investigations.

References

Aguilar-Martínez, J. A., Poza-Carrión, C., and Cubas, P. (2007). *Arabidopsis* BRANCHED1 acts as an integrator of branching signals within axillary buds. *The Plant Cell* **19**, 458-472.

Ahmad, M. S. A., Hussain, M., Saddiq, R., and Alvi, A. K. (2007). Mungbean: a nickel indicator, accumulator or excluder? *Bulletin of Environmental Contamination and Toxicology* **78**, 319-324.

Ahsan, N., Nakamura, T., and Komatsu, S. (2012). Differential responses of microsomal proteins and metabolites in two contrasting cadmium (Cd)-accumulating soybean cultivars under Cd stress. *Amino Acids* **42**, 317-327.

Akaneme, F. I., and Ene-Obong, E. E. (2008). Tissue culture in *Pinus caribaea* Mor. var. Hondurensis barr. and golf. II: Effects of two auxins and two cytokinins on callus growth habits and subsequent organogenesis. *African Journal of Biotechnology* **7**, 757-765.

Albiski, F., Najla, S., Sanoubar, R., Alkabani, N., and Murshed, R. (2012). *In vitro* screening of potato lines for drought tolerance. *Physiology and Molecular Biology of Plants* **18**, 315-321.

Alexander, P. D., Alloway, B. J., and Dourado, A. M. (2006). Genotypic variations in the accumulation of Cd, Cu, Pb and Zn exhibited by six commonly grown vegetables. *Environmental Pollution* **144**, 736-745.

Alscher, R. G., Erturk, N., and Heath, L. S. (2002). Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. *Journal of Experimental Botany* **53**, 1331-1341.

Andersson, A., and Pettersson, O. (1981). Cadmium in swedish winter wheat. Regional differences and their origin. *Swedish Journal of Agricural Research* **11**, 49-55.

Arao, T., and Ae, N. (2001). Screening of genotypes with low cadmium content in soybean seed and rice grains. *Developments in Plant and Soil Sciences* **92**, 292-293.

Arao, T., and Ae, N. (2003). Genotypic variations in cadmium levels of rice grain. *Soil Science and Plant Nutrition* **49**, 473-479.

Arao, T., Ae, N., Sugiyama, M., and Takahashi, M. (2003). Genotypic differences in cadmium uptake and distribution in soybeans. *Plant and Soil* **251**, 247-253.

Arao, T., and Ishikawa, S. (2006). Genotypic differences in cadmium concentration and distribution of soybean and rice. *Japan Agricultural Research Quarterly* **40**, 21-30.

Ashraf, M., and Harris, P. J. C. (2013). Photosynthesis under stressful environments: An overview. *Photosynthetica* **51**, 163-190.

Ashrafzadeh, S., and Leung, D. W. M. (2015). Microtuber formation in potato callus. *Scienceasia* **41**, 1-4.

Atwell, B. J., Kriedemann, P. E., and Turnbull, C. G. N. (1999). "Plants in action: adaptation in nature, performance in cultivation," Macmillan Education Australia, Melbourne.

Babula, P., Adam, V., Havel, L., and Kizek, R. (2012). Cadmium accumulation by plants of Brassicaceae family and its connection with their primary and secondary metabolism. *Environmental Pollution* **21**, 71-97.

Bairu, M., Aremu, A., and Van Staden, J. (2011). Somaclonal variation in plants: causes and detection methods. *Plant Growth Regulators* **63**, 147-173.

Baisakhi, B., Patra, J., Panigrahy, R., and Panda, B. (2003). Differential antioxidant enzyme and thiol responses of tolerant and non-tolerant clones of *Chloris barbata* to cadmium-stress. *Acta Physiologiae Plantarum* **25**, 357-363.

Bajaj, Y. P. S. (1990). Somaclonal Variation — Origin, induction, cryopreservation, and implications in plant breeding. *In* "Somaclonal Variation in Crop Improvement I" (Y. P. S. Bajaj, ed.), Vol. 11, pp. 3-48. Springer Berlin Heidelberg.

Bates, L. S., Waldren, R. P., and Teare, I. D. (1973). Rapid determination of free proline for water-stress studies. *Plant and Soil* **39**, 205-207.

Bell, M. J., McLaughlin, M. J., Wright, G. C., and Cruickshank, A. (1997). Inter- and intra-specific variation in accumulation of cadmium by peanut, soybean, and navybean. *Australian Journal of Agricultural Research* **48**, 1151-1160.

Belleghem, F. V., Cuypers, A., Semane, B., Smeets, K., Vangronsveld, J., d'Haen, J., and Roland, V. (2007). Subcellular localization of cadmium in roots and leaves of *Arabidopsis thaliana*. *New Phytologist* **173**, 495-508.

Benavides, M. P., Gallego, S. M., and Tomaro, M. L. (2005). Cadmium toxicity in plants. *Brazilian Journal of Plant Physioly* **17**, 21-34.

Bhaduri, A., and Fulekar, M. H. (2012). Antioxidant enzyme responses of plants to heavy metal stress. *Reviews in Environmental Science and Biotechnology* **11**, 55-69.

Bočová, B., Huttová, J., Liptáková, Ľ., Mistrík, I., Ollé, M., and Tamás, L. (2012). Impact of short-term cadmium treatment on catalase and ascorbate peroxidase activities in barley root tips. *Biologia Plantarum* **56**, 724-728.

Brune, A., Urbach, W., and Dietz, K. J. (1994). Compartmentation and transport of zinc in barley primary leaves as basic mechanisms involved in zinc tolerance. *Plant, Cell & Environment* **17**, 153-162.

Brunetti, P., Zanella, L., De Paolis, A., Di Litta, D., Cecchetti, V., Falasca, G., Barbieri, M., Altamura, M. M., Costantino, P., and Cardarelli, M. (2015). Cadmium-inducible expression of the ABC-type transporter AtABCC3 increases phytochelatin-mediated cadmium tolerance in Arabidopsis. *Journal of Experimental Botany* **66**, 3815-3829.

CadmiumWorkingGroup (2008). Report one: cadmium in New Zealand agriculture. *In* "Ministry of Agriculture and Forestry".

Callender, E. (2003). Heavy metals in the environment—Historical trends. *In* "Treatise on Geochemistry" (H. D. Holland and K. K. Turekian, eds.), pp. 67-105. Pergamon, Oxford.

Carrier, P., Baryla, A., and Havaux, M. (2003). Cadmium distribution and microlocalization in oilseed rape (*Brassica napus*) after long-term growth on cadmium-contaminated soil. *Planta* **216**, 939-950.

Cataldo, D. A., and Garland, T. R. (1983). Cadmium uptake kinetics in intact soybean plants. *Plant Physiology* **73**, 844-848.

Chen, F., Dong, J., Wang, F., Wu, F., Zhang, G., Li, G., Chen, Z., Chen, J., and Wei, K. (2007). Identification of barley genotypes with low grain Cd accumulation and its interaction with four microelements. *Chemosphere* **67**, 2082-2088.

Chen, L., Guo, Y., Yang, L., and Wang, Q. (2008). Synergistic defensive mechanism of phytochelatins and antioxidative enzymes in *Brassica chinensis* L. against Cd stress. *Chinese Science Bulletin* **53**, 1503-1511.

Chen, Y., Li, T.-q., Han, X., Ding, Z.-l., Yang, X.-e., and Jin, Y.-f. (2012). Cadmium accumulation in different pakchoi cultivars and screening for pollution-safe cultivars. *Journal of Zhejiang University SCIENCE B* **13**, 494-502.

Chen, Z., Zhao, Y., Gu, L., Wang, S., Li, Y., and Dong, F. (2014). Accumulation and localization of cadmium in potato (*Solanum tuberosum*) under different soil Cd levels. *Bulletin of Environmental Contamination and Toxicology* **92**, 745-751.

Cheng, W.-d., Zhang, G.-p., Yao, H.-g., Wu, W., and Xu, M. (2006). Genotypic and environmental variation in cadmium, chromium, arsenic, nickel, and lead concentrations in rice grains. *Journal of Zhejiang University SCIENCE B* **7**, 565-571.

Cherif, J., Derbel, N., Nakkach, M., Von Bergmann, H., Jemal, F., and Lakhdar, Z. B. (2012). Spectroscopic studies of photosynthetic responses of tomato plants to the interaction of zinc and cadmium toxicity. *Journal of Photochemistry and Photobiology B: Biology* **111**, 9-16.

Chin, L. (2007). Investigations into lead (Pb) accumulation in *Symphytum officinale* L: a phytoremediation study : a thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Biotechnology.

Cho, U.-H., and Seo, N.-H. (2005). Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. *Plant Science* **168**, 113-120.

Cieslinski, G., Van Rees, K. C. J., Huang, P. M., Kozak, L. M., Rostad, H. P. W., and Knott, D. R. (1996). Cadmium uptake and bioaccumulation in selected cultivars of durum wheat and flax as affected by soil type. *Plant and Soil* **182**, 115-124.

Clarke, J. M., Leisle, D., and Kopytko, G. L. (1997). Inheritance of cadmium concentration in five durum wheat crosses. *Crop Science* **37**, 1722-1726.

Clemens, S. (2001). Molecular mechanisms of plant metal tolerance and homeostasis. *Planta* **212**, 475-486.

Cosio, C., Vollenweider, P., and Keller, C. (2006). Localization and effects of cadmium in leaves of a cadmium-tolerant willow (*Salix viminalis* L.): I. Macrolocalization and phytotoxic effects of cadmium. *Environmental and Experimental Botany* **58**, 64-74.

Crews, H. M., and Davies, B. E. (1985). Heavy metal uptake from contaminated soils by six varieties of lettuce (*Lactuca sativa* L.). *The Journal of Agricural Science* **105**, 591-595.

Crupper, S. S., Worrell, V., Stewart, G. C., and Iandolo, J. J. (1999). Cloning and expression of cadD, a new cadmium resistance gene of *Staphylococcus aureus*. *Journal of Bacteriology* **181**, 4071-4075.

Cuypers, A., Plusquin, M., Remans, T., Jozefczak, M., Keunen, E., Gielen, H., Opdenakker, K., Nair, A., Munters, E., Artois, T., Nawrot, T., Vangronsveld, J., and Smeets, K. (2010). Cadmium stress: an oxidative challenge. *BioMetals* **23**, 927-940.

Czarnecka, E., Edelman, L., Schoeffl, F., and Key, J. L. (1984). Comparative analysis of physical stress responses in soybean seedlings using cloned heat shock cDNAs. *Plant Molecular Biology* **3**, 45-58.

Czarnecka, E., Nagao, R. T., Key, J. L., and Gurley, W. B. (1988). Characterization of *Gmhsp26-A*, a stress gene encoding a divergent heat shock protein of soybean: heavy-metal-induced inhibition of intron processing. *Molecular and Cellular Biology* **8**, 1113-1122.

Daghan, H., Arslan, M., Uygur, V., and Koleli, N. (2013). Transformation of tobacco with *ScMTII* gene-enhanced cadmium and zinc accumulation. *Clean - Soil, Air, Water* **41**, 503-509.

Das, P., Samantaray, S., and Rout, G. R. (1998). Studies on cadmium toxicity in plants: a review. *Environmental Pollution* **98**, 29-36.

Dauthieu, M., Denaix, L., Nguyen, C., Panfili, F., Perrot, F., and Potin-Gautier, M. (2009). Cadmium uptake and distribution in *Arabidopsis thaliana* exposed to low chronic concentrations depends on plant growth. *Plant and Soil* **322**, 239-249.

Demiral, T., and Türkan, İ. (2005). Comparative lipid peroxidation, antioxidant defense systems and proline content in roots of two rice cultivars differing in salt tolerance. *Environmental and Experimental Botany* **53**, 247-257.

Domazlicka, E., and Opatrny, Z. (1989). The effect of cadmium on potato (*Solanum tuberosum* L.) shoot culture growth. *Biologia Plantarum* **31**, 408-412.

Douchiche, O., Soret-Morvan, O., Chaïbi, W., Morvan, C., and Paynel, F. (2010). Characteristics of cadmium tolerance in 'Hermes' flax seedlings: Contribution of cell walls. *Chemosphere* **81**, 1430-1436.

Drążkiewicz, M., Skórzyńska-Polit, E., and Krupa, Z. (2007). The redox state and activity of superoxide dismutase classes in *Arabidopsis thaliana* under cadmium or copper stress. *Chemosphere* **67**, 188-193.

Du Plessis, R., and Hindmarsh, R. (2008). GMO regulation and civic participation at the "edge of the world": the case of Australia and New Zealand. *New Genetics and Society* **27**, 181-199.

Dunbar, K. R., McLaughlin, M. J., and Reid, R. J. (2003). The uptake and partitioning of cadmium in two cultivars of potato (*Solanum tuberosum* L.). *Journal of Experimental Botany* **54**, 349-354.

Eapen, S., and D'Souza, S. F. (2005). Prospects of genetic engineering of plants for phytoremediation of toxic metals. *Biotechnology Advances* **23**, 97-114.

Edelman, L., Czarnecka, E., and Key, J. L. (1988). Induction and accumulation of heat shock-specific poly(A+) RNAs and proteins in soybean seedlings during arsenite and cadmium treatments. *Plant Physiology* **86**, 1048-1056.

Eriksson, J. (1990). Effects of nitrogen-containing fertilizers on solubility and plant uptake of cadmium. *Water, Air, and Soil Pollution* **49**, 355-368.

Ernst, W. H. O. (2006). Evolution of metal tolerance in higher plants. *Forest, Snow and Landscape Research* **80**, 251-274.

Fan, J. L., Ziadi, N., Belanger, G., Parent, L. E., Cambouris, A., and Hu, Z.-Y. (2009). Cadmium accumulation in potato tubers produced in Quebec. *Canadian Journal of Soil Science* **89**, 435-443.

Food Standards Australia New Zealand (2011). "Food standards."

Fornazier, R., Ferreira, R., Pereira, G. G., Molina, S. G., Smith, R. J., Lea, P., and Azevedo, R. (2002). Cadmium stress in sugar cane callus cultures: Effect on antioxidant enzymes. *Plant Cell, Tissue and Organ Culture* **71**, 125-131.

Furini, A. (2012). "Plants and heavy metals," Springer.

Gallego, S., Pena, L., Barcia, R., CB, A., Iannone, M., and Rosales, E. (2012). Unravelling cadmium toxicity and tolerance in plants: Insight into regulatory mechanisms. *Environmental and Experimental Botany* **83**, 33-46.

Gao, X., Mohr, R. M., McLaren, D. L., and Grant, C. A. (2011). Grain cadmium and zinc concentrations in wheat as affected by genotypic variation and potassium chloride fertilization. *Field Crops Research* **122**, 95-103.

Gaspar, T., Kevers, C., Penel, C., Greppin, H., Reid, D. M., and Thorpe, T. A. (1996). Plant hormones and plant growth regulators in plant tissue culture. *In Vitro Cellular & Developmental Biology - Plant* **32**, 272-289.

Gaw, S. K., Wilkins, A. L., Kim, N. D., Palmer, G. T., and Robinson, P. (2006). Trace element and ΣDDT concentrations in horticultural soils from the Tasman, Waikato and Auckland regions of New Zealand. *Science of The Total Environment* **355**, 31-47.

Gill, S. S., and Tuteja, N. (2010). Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. *Plant Physiology and Biochemistry* **48**, 909-930.

Gill, S. S., and Tuteja, N. (2011). Cadmium stress tolerance in crop plants: probing the role of sulfur. *Plant Signaling and Behavior* **6**, 215-222.

Göhre, V., and Paszkowski, U. (2006). Contribution of the arbuscular mycorrhizal symbiosis to heavy metal phytoremediation. *Planta* **223**, 1115-1122.

Golan-Goldhirsh, A., Barazani, O., Nepovim, A., Soudek, P., Smrcek, S., Dufkova, L., Krenkova, S., Yrjala, K., Schröder, P., and Vanek, T. (2004). Plant response to heavy metals and organic pollutants in cell culture and at whole plant level. *Journal of Soils and Sediments* **4**, 133-140.

Goldsbrough, P. B. (1991). Phytochelatin accumulation and cadmium tolerance in selected tomato cell lines. *Plant Physiology* **97**, 306-312.

Gonçalves, J. F., Tabaldi, L. A., Cargnelutti, D., Pereira, L. B., Maldaner, J., Becker, A. G., Rossato, L. V., Rauber, R., Bagatini, M. D., Bisognin, D. A., Schetinger, M. R., and Nicoloso, F. T. (2009). Cadmium-induced oxidative stress in two potato cultivars. *Biometals* **22**, 779-792.

Gopal, J., and Iwama, K. (2007). *In vitro* screening of potato against water-stress mediated through sorbitol and polyethylene glycol. *Plant Cell Reports* **26**, 693-700.

Gorinova, N., Nedkovska, M., Todorovska, E., Simova-Stoilova, L., Stoyanova, Z., Georgieva, K., Demirevska-Kepova, K., Atanassov, A., and Herzig, R. (2007). Improved phytoaccumulation of cadmium by genetically modified tobacco plants (*Nicotiana tabacum* L.). Physiological and biochemical response of the transformants to cadmium toxicity. *Environmental Pollution* **145**, 161-170.

Grant, C. A., Clarke, J. M., Duguid, S., and Chaney, R. L. (2008). Selection and breeding of plant cultivars to minimize cadmium accumulation. *The Science of the Total Environment* **390**, 301-310.

Gray, C. W., and McLaren, R. G. (2005). The effect of ryegrass variety on trace metal uptake. *New Zealand Journal of Agricultural Research* **48**, 285-292.

Gray, C. W., McLaren, R. G., and Roberts, A. H. C. (2001). Cadmium concentrations in some New Zealand wheat grain. *New Zealand Journal of Crop and Horticultural Science* **29**, 125-136.

Greger, M., and Loefstedt, M. (2004). Comparison of uptake and distribution of cadmium in different cultivars of bread and durum wheat. *Crop Science* **44**, 501-507.

Grotz, N., and Guerinot, M. L. (2006). Molecular aspects of Cu, Fe and Zn homeostasis in plants. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* **1763**, 595-608.

Guo, X.-F., Wei, Z.-B., Wu, Q.-T., Qiu, J.-R., and Zhou, J.-L. (2011). Cadmium and zinc accumulation in maize grain as affected by cultivars and chemical fixation amendments. *Pedosphere* **21**, 650-656.

Gzyl, J., and Gwóźdź, E. (2005). Selection *in vitro* and accumulation of phytochelatins in cadmium tolerant cell line of cucumber (*Cucumis sativus*). *Plant Cell, Tissue and Organ Culture* **80**, 59-67.

Hagen, S., LeTourneau, D., Muneta, P., and Brown, J. (1990). Initiation and culture of potato tuber callus tissue with picloram. *Plant Growth Regulation* **9**, 341-345.

Hall, J. L. (2002). Cellular mechanisms for heavy metal detoxification and tolerance. *Journal of Experimental Botany* **53**, 1-11.

Hall, J. L., and Williams, L. E. (2003). Transition metal transporters in plants. *Journal of Experimental Botany* **54**, 2601-2613.

Halmer, P., and Thorpe, T. A. (1976). Kinetin induced changes in cell wall composition of tobacco callus. *Phytochemistry* **15**, 1585-1588.

Hamer, D. H. (1986). Metallothionein. *Annual Review of Biochemistry* **55**, 913-951.

Hansen, J., Nielsen, B., and Nielsen, S. V. S. (1999). *In vitro* shoot regeneration of *Solanum tuberosum* cultivars: Interactions of medium composition and leaf, leaflet, and explant position. *Potato Research* **42**, 141-151.

Harris, M. R., Harrison, S. J., Wilson, N. J., and Lepp, N. W. (1981). Varietal differences in trace metal partitioning by six potato cultivars. pp. 399-402. CEP Consult. Ltd.

Hartke, S., Silva, A., and Moraes, M. (2013). Cadmium accumulation in tomato cultivars and its effect on expression of metal transport-related genes. *Bulletin of Environmental Contamination and Toxicology* **90**, 227-232.

Hayat, S., Hayat, Q., Alyemeni, M. N., Wani, A. S., Pichtel, J., and Ahmad, A. (2012). Role of proline under changing environments: A review. *Plant Signaling & Behavior* **7**, 1456-1466.

Haynes, K. G., Yencho, G. C., Clough, M. E., Henninger, M. R., and Sterrett, S. B. (2012). Genetic variation for potato tuber micronutrient content and implications for biofortification of potatoes to reduce micronutrient malnutrition. *American Journal of Potato Research* **89**, 192-198.

He, J., Zhu, C., Ren, Y., Yan, Y., and Jiang, D. (2006). Genotypic variation in grain cadmium concentration of lowland rice. *Journal of Plant Nutrition and Soil Science* **169**, 711-716.

He, S., Han, Y., Wang, Y., Zhai, H., and Liu, Q. (2009). *In vitro* selection and identification of sweetpotato (*Ipomoea batatas* (L.) Lam.) plants tolerant to NaCl. *Plant Cell, Tissue and Organ Culture* **96**, 69-74.

Hell, R. (2003). Transgenic plants and crops. *Journal of Plant Physiology* **160**, 706-718.

Heppner, C., Åkesson, A., Amzal, B., Di Domenico, A., Dorne, J. L. C. M., Cristoph, E. H., Galli, C. L., Grandjean, P., Järup, L., Gzyl, J., Lindtner, O., Mutti, A., Nordberg, G., Venäläinen, E. R., and Dogliotti, E. (2009). Risk assessment of cadmium in food: Implications for human health. *Toxicology Letters* **189**, 233-233.

Hocking, P. J., and McLaughlin, M. J. (2000). Genotypic variation in cadmium accumulation by seed of linseed, and comparison with seeds of some other crop species. *Australian Journal of Agricultural Research* **51**, 427-433.

Hoen, P. A. C., Van der Lans, C. A. C., Van Eck, M., Bijsterbosch, M. K., Van Berkel, T. J. C., and Twisk, J. (2003). Aorta of ApoE-deficient mice responds to atherogenic stimuli by a prelesional increase and subsequent decrease in the expression of antioxidant enzymes. *Circulation Research* **93**, 262-269.

Hongbo, S., Liye, C., Gang, X., Kun, Y., Lihua, Z., and Junna, S. (2011). Progress in phytoremediating heavy-metal contaminated soils. *In* "Detoxification of Heavy Metals" (I. Sherameti and A. Varma, eds.), Vol. 30, pp. 73-90. Springer Berlin Heidelberg.

Hongjiang, Z., Xizhou, Z., Tingxuan, L., and Fu, H. (2014). Variation of cadmium uptake, translocation among rice lines and detecting for potential cadmium-safe cultivars. *Environmental Earth Sciences* **71**, 277-286.

Howden, R., and Cobbett, C. S. (1992). Cadmium-sensitive mutants of *Arabidopsis thaliana*. *Plant Physiology* **100**, 100-107.

Huang, J., Zhang, P.-J., Zhang, J., Lu, Y.-B., Huang, F., and Li, M.-J. (2013). Chlorophyll content and chlorophyll fluorescence in tomato leaves infested with an invasive mealybug, *Phenacoccus solenopsis* (Hemiptera: Pseudococcidae). *Environmental Entomology* **42**, 973-979.

Ikeuchi, M., Sugimoto, K., and Iwase, A. (2013). Plant callus: mechanisms of induction and repression. *The Plant Cell* **25**, 3159-3173.

Iori, V., Pietrini, F., Massacci, A., and Zacchini, M. (2012). Induction of metal binding compounds and antioxidative defence in callus cultures of two black poplar (*P. nigra*) clones with different tolerance to cadmium. *Plant Cell, Tissue and Organ Culture* **108**, 17-26.

Ishikawa, S., Ae, N., and Yano, M. (2005). Chromosomal regions with quantitative trait loci controlling cadmium concentration in brown rice (*Oryza sativa*). *New Phytologist* **168**, 345-350.

Jarvis, M. D. (2001). An investigation into some aspects related to the phytoremediation potential of *Pinus radiata* (D. Don) & *Chamaecytisus proliferus* (L.f.) Link ssp. proliferus var. palmensis (H. Christ.): a thesis

submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Biotechnology in the University of Canterbury.

JayaSree, T., Pavan, U., Ramesh, M., Rao, A. V., Jagan Mohan Reddy, K., and Sadanandam, A. (2001). Somatic embryogenesis from leaf cultures of potato. *Plant Cell, Tissue and Organ Culture* **64**, 13-17.

Joseph, P. (2009). Mechanisms of cadmium carcinogenesis. *Toxicology and Applied Pharmacology* **238**, 272-279.

Jouili, H., Bouazizi, H., and El Ferjani, E. (2011). Plant peroxidases: biomarkers of metallic stress. *Acta Physiologiae Plantarum* **33**, 2075-2082.

Kaeppler, S., Kaeppler, H., and Rhee, Y. (2000). Epigenetic aspects of somaclonal variation in plants. *Plant Molecular Biology* **43**, 179-188.

Karp, A. (1990). Somaclonal variation in potato. *In* "Somaclonal Variation in Crop Improvement I" (Y. P. S. Bajaj, ed.), Vol. 11, pp. 379-399. Springer Berlin Heidelberg.

Khan, M. D., Mei, L., Ali, B., Chen, Y., Cheng, X., and Zhu, S. J. (2013). Cadmium-induced upregulation of lipid peroxidation and reactive oxygen species caused physiological, biochemical, and ultrastructural changes in upland cotton seedlings. *BioMed Research International* **2013**, 1-10.

Khatun, N., Bari, M. A., Islam, R., Huda, S., Siddique, N. A., Rahman, M. H., and Mollah, M. U. (2003). Callus induction and regeneration from nodal segment of potato cultivar Diamant. *Journal of Biological Sciences* **3**, 1101-1106.

Kim, N. (2005). "Cadmium accumulation in Waikato soils," Environment Waikado Regional Council. 51.

Kjellstrom, T., Linnman, L., and Elinder, C. (1975). Variation of cadmium concentration in Swedish wheat and barley. An indicator of changes in daily cadmium intake during the 20th century. *Archives of Environmental Health* **30**, 321-328.

Klotz, K., Weistenhöfer, W., and Drexler, H. (2013). Determination of cadmium in biological samples. *In* "Cadmium: From Toxicity to Essentiality" (A. Sigel, H. Sigel and R. K. O. Sigel, eds.), Vol. 11, pp. 85-98. Springer Netherlands.

Koca, H., Bor, M., Ozdemir, F., and Turkan, I. (2007). The effect of salt stress on lipid peroxidation, antioxidative enzymes and proline content of sesame cultivars. *Environmental and Experimental Botany* **60**, 344-351.

Lachman, J., Hamouz, K., Šulc, M., Orsák, M., Pivec, V., Hejtmánková, A., Dvořák, P., and Čepl, J. (2009). Cultivar differences of total anthocyanins and anthocyanidins in red and purple-fleshed potatoes and their relation to antioxidant activity. *Food Chemistry* **114**, 836-843.

Larsson Jönsson, E., and Asp, H. (2011). Influence of nitrogen supply on cadmium accumulation in potato tubers. *Journal of Plant Nutrition* **34**, 345-360.

Lefevre, I., Correal, E., and Lutts, S. (2005). Cadmium tolerance and accumulation in the noxious weed *Zygophyllum fabago*. *Canadian Journal of Botany* **83**, 1655-1655.

Lei, Y., Korpelainen, H., and Li, C. (2007). Physiological and biochemical responses to high Mn concentrations in two contrasting *Populus cathayana* populations. *Chemosphere* **68**, 686-694.

Lestari, E., and Endang Gati, L. (2006). Review: *In vitro* selection and somaclonal variation for biotic and abiotic stress tolerance. *Biodiversitas* **7**, 297-301.

Leung, D. W. M., ed. (2013). "Recent advances towards improved phytoremediation of heavy metal pollution," Vol. 150, Dubai: Bentham Sciences Publishers.

Li, X., Zhou, Q., Wei, S., and Ren, W. (2012). Identification of cadmium-excluding welsh onion (*Allium fistulosum* L.) cultivars and their mechanisms of low cadmium accumulation. *Environmental Science and Pollution Research* **19**, 1773-1780.

Li, Y.-M., Chaney, R., Schneiter, A., Miller, J., Elias, E., and Hammond, J. (1997). Screening for low grain cadmium phenotypes in sunflower, durum wheat and flax. *Euphytica* **94**, 23-30.

Liners, F., Gaspar, T., and Van Cutsem, P. (1994). Acetyl- and methyl-esterification of pectins of friable and compact sugar-beet calli: consequences for intercellular adhesion. *Planta* **192**, 545-556.

Liu, J., Zhu, Q., Zhang, Z., Xu, J., Yang, J., and Wong, M. H. (2005). Variations in cadmium accumulation among rice cultivars and types and the selection of cultivars for reducing cadmium in the diet. *Journal of the Science of Food and Agriculture* **85**, 147-153.

Liu, W., Zhou, Q., An, J., Sun, Y., and Liu, R. (2010). Variations in cadmium accumulation among Chinese cabbage cultivars and screening for Cd-safe cultivars. *Journal of Hazardous Materials* **173**, 737-743.

Lyubenova, L., and Schroeder, P. (2010). Uptake and effect of heavy metals on the plant detoxification cascade in the presence and absence of organic pollutants. *Soil Biolgy and Biochemistry* **19**, 65-85.

Madan, S., Nainawatee, H. S., Jain, R. K., and Chowdhury, J. B. (1995). Proline and proline metabolizing enzymes in *in-vitro* selected NaCl-tolerant *Brassica-juncea* L under salt stress. *Annals of Botany* **76**, 51-57.

Marmiroli, M., Imperiale, D., Maestri, E., and Marmiroli, N. (2013). The response of *Populus* spp. to cadmium stress: Chemical, morphological and proteomics study. *Chemosphere* **93**, 1333-1344.

Martins, L., Mourato, M., Baptista, S., Reis, R., Carvalheiro, F., Almeida, A., Fevereiro, P., and Cuypers, A. (2014). Response to oxidative stress induced by cadmium and copper in tobacco plants (*Nicotiana tabacum*) engineered with the trehalose-6-phosphate synthase gene (*AtTPS1*). *Acta Physiologiae Plantarum* **36**, 755-765.

Martins, L. L., Reis, R., Moreira, I., Pinto, F., Sales, J., and Mourato, M. P. (2013). Antioxidative response of plants to oxidative stress induced by cadmium. pp. 87-112. Nova Science Publishers, Inc.

McDowell, R. W., Taylor, M. D., and Stevenson, B. A. (2013). Natural background and anthropogenic contributions of cadmium to New Zealand soils. *Agriculture, Ecosystem and Environment* **165**, 80-87.

McLaughlin, M. J., Bell, M. J., Wright, G. C., and Cozens, G. D. (2000). Uptake and partitioning of cadmium by cultivars of peanut (*Arachis hypogaea* L.). *Plant and Soil* **222**, 51-58.

McLaughlin, M. J., Maier, N. A., Rayment, G. E., Sparrow, L. A., Berg, G., McKay, A., Milham, P., Merry, R. H., and Smart, M. K. (1997). Cadmium in Australian potato tubers and soils. *Journal of environmental quality* **26**, 1644-1649.

McLaughlin, M. J., Parker, D. R., and Clarke, J. M. (1999). Metals and micronutrients – food safety issues. *Field Crops Research* **60**, 143-163.

McLaughlin, M. J., Williams, C. M. J., McKay, A., Kirkham, R., Gunton, J., Jackson, K. J., Thompson, R., Dowling, B., Partington, D., and et, a.

(1994). Effect of cultivar on uptake of cadmium by potato tubers. *Australian journal of agricultural research* **45**, 1483-1495.

Ministry of Agriculture and Foresty (2009). "New Zealand total diet study ".

Ministry of Health (2012). "A focus on pacific nutrition: findings from the 2008/09 New Zealand adult nutrition survey," Wellington: Ministry of Health.

Mishra, S., Srivastava, S., Tripathi, R. D., Govindarajan, R., Kuriakose, S. V., and Prasad, M. N. V. (2006). Phytochelatin synthesis and response of antioxidants during cadmium stress in *Bacopa monnieri* L. *Plant Physiology and Biochemistry* **44**, 25-37.

Mittler, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* **7**, 405-410.

Mohan Jain, S. (2001). Tissue culture-derived variation in crop improvement. *Euphytica* **118**, 153-166.

Mullins, M. (1987). Subcellular localization of metal s in metal tolerant higher plants.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tabacco tissue cultures. *Physiologia Plantarum* **15**, 473-497.

Nadgórska-Socha, A., Ptasiński, B., and Kita, A. (2013). Heavy metal bioaccumulation and antioxidative responses in *Cardaminopsis arenosa* and *Plantago lanceolata* leaves from metalliferous and non-metalliferous sites: a field study. *Ecotoxicology* **22**, 1422-1434.

Nazar, R., Iqbal, N., Masood, A., Khan, M. I. R., Sveed, S., and Khan, N. A. (2012). Cadmium toxicity in plants and role of mineral nutrients in its alleviation. *American Journal of Plant Science* **3**, 1476-1489.

Nehnevajova, E., Herzig, R., Erismann, K.-H., and Schwitzguébel, J.-P. (2007). *In vitro* breeding of *Brassica juncea* L. to enhance metal accumulation and extraction properties. *Plant Cell Reports* **26**, 429-437.

Ni, C.-y., Chen, Y.-x., Lin, Q., and Tian, G.-m. (2005). Subcellular localization of copper in tolerant and non-tolerant plant. *Journal of Environmental Sciences* **17**, 452-456.

Nocito, F. F., Lancilli, C., Dendena, B., Lucchini, G., and Sacchi, G. A. (2011). Cadmium retention in rice roots is influenced by cadmium availability, chelation and translocation. *Plant, Cell and Environment* **34**, 994-1008.

Oliver, D. P., Gartrell, J. W., Tiller, K. G., Correll, R., Cozens, G. D., and Youngberg, B. L. (1995). Differential responses of Australian wheat cultivars to cadmium concentration in wheat grain. *Australian journal of agricultural research* **46**, 873-886.

Olowoyo, J. O., Okedeyi, O. O., Mkolo, N. M., Lion, G. N., and Mdakane, S. T. R. (2012). Uptake and translocation of heavy metals by medicinal plants growing around a waste dump site in Pretoria, South Africa. *South African Journal of Botany* **78**, 116-121.

Olowoyo, J. O., van Heerden, E., Fischer, J. L., and Baker, C. (2010). Trace metals in soil and leaves of *Jacaranda mimosifolia* in Tshwane area, South Africa. *Atmospheric Environment* **44**, 1826-1830.

Oporto, C., Vandecasteele, C., and Smolders, E. (2007). Elevated cadmium concentrations in potato tubers due to irrigation with river water contaminated by mining in potosí, Bolivia. *Journal of environmental quality* **36**, 1181-1186.

Ovečka, M., and Takáč, T. (2014). Managing heavy metal toxicity stress in plants: Biological and biotechnological tools. *Biotechnology Advances* **32**, 73-86.

Ozturk, E., Atsan, E., Polat, T., and Kara, K. (2011). Variation in heavy metal concentrations of potato *(Solanum tuberosum* L.) cultivars. *Journal of Animal and Plant Sciences* **21**, 235-239.

Pan, J., Plant, J., Voulvoulis, N., Oates, C., and Ihlenfeld, C. (2010). Cadmium levels in Europe: implications for human health. *Environal Geochemistry and Health* **32**, 1-12.

Perez-Clemente, R. M., Vives, V., Zandalinas, S. I., Lopez-Climent, M. F., Munoz, V., and Gomez-Cadenas, A. (2013). Biotechnological approaches to study plant responses to stress. *BioMed Research International* **2013**, 1-10.

Phang, I. C. (2010). Response of *Arabidopsis thaliana* seedlings to lead exposure: a thesis submitted in partial fulfilment of the requirement for the degree of Doctor of Philosophy at the University of Canterbury.

Phang, I. C., Leung, D. W. M., Taylor, H. H., and Burritt, D. J. (2011). Correlation of growth inhibition with accumulation of Pb in cell wall and changes in response to oxidative stress in *Arabidopsis thaliana* seedlings. *Plant Growth Regulator* **64**, 17-25.

Pilon-Smits, E. (2005). Phytoremediation. *Annual review of plant biology* **56**, 15-39.

Piotto, F. A., Tulmann-Neto, A., Franco, M. R., Boaretto, L. F., and Azevedo, R. A. (2014). Rapid screening for selection of heavy metal-tolerant plants. *Crop Breeding and Applied Biotechnology* **14**, 1-7.

Prasad, M. N. V., and Hagemeyer, J. r. (1999). "Heavy metal stress in plants: from molecules to ecosystems," Springer, New York; Berlin.

Predieri, S. (2001). Mutation induction and tissue culture in improving fruits. *Plant Cell, Tissue and Organ Culture* **64**, 185-210.

Qi-Tang W. U. , Zhao-Li Xu, Hui YE, and C., W. J. W. (2007). Chemical composition of root and stem saps in relation to cadmium resistance and accumulation in *Brassica parachinensis*. *Pedosphere* **17**, 352-359.

Qiu, Q., Wang, Y., Yang, Z., Xin, J., Yuan, J., Wang, J., and Xin, G. (2011). Responses of different Chinese flowering cabbage (*Brassica parachinensis* L.) cultivars to cadmium and lead exposure: screening for Cd + Pb pollution-safe cultivars. *CLEAN – Soil, Air, Water* **39**, 925-932.

Rahman, M., Islam, R., Hossain, M., and Haider, S. (2009). "Differential response of potato under sodium chloride stress conditions *in vitro*."

Rai, M. K., Kalia, R. K., Singh, R., Gangola, M. P., and Dhawan, A. K. (2011). Developing stress tolerant plants through in vitro selection—An overview of the recent progress. *Environmental and Experimental Botany* **71**, 89-98.

Ranieri, A., Castagna, A., Lorenzini, G., and Soldatini, G. F. (1997). Changes in thylakoid protein patterns and antioxidant levels in two wheat cultivars with different sensitivity to sulfur dioxide. *Environmental and Experimental Botany* **37**, 125-135.

Reid, R. J., Dunbar, K. R., and McLaughlin, M. J. (2003). Cadmium loading into potato tubers: The roles of the periderm, xylem and phloem. *Plant, Cell and Environment* **26**, 201-206.

Ridge, I. (1997). Oxidative stress and antioxidant defenses in biology. *Mycologist* **11**, 44-45.

Roustan, J. P., Latche, A., and Fallot, J. (1989). Stimulation of *Daucus-carota* somatic embryogenesis by inhibitors of ethylen synthesis-cobalt and nickel. *Plant Cell Reports* **8**, 182-185.

Rout, G. R., and Sahoo, S. (2007). *In vitro* selection and plant regeneration of copper-tolerant plants from leaf explants of *Nicotiana tabacum* L. cv. 'Xanthi'. *Plant Breeding* **126**, 403-409.

Rout, G. R., Samantaray, S., and Das, P. (1998). The role of nickel on somatic embryogenesis in *Setaria italica* L. *in vitro*. *Euphytica* **101**, 319-324.

Rout, G. R., Samantaray, S., and Das, P. (1999). *In vitro* selection and biochemical characterisation of zinc and manganese adapted callus lines in *Brassica* spp. *Plant Science* **146**, 89-100.

Roy, B., and Mandal, A. (2005). Towards development of Al-toxicity tolerant lines in indica rice by exploiting somaclonal variation. *Euphytica* **145**, 221-227.

Saito, A., Saito, M., Ichikawa, Y., Yoshiba, M., Tadano, T., Miwa, E., and Higuchi, K. (2010). Difference in the distribution and speciation of cellular nickel between nickel-tolerant and non-tolerant *Nicotiana tabacum* L. cv. BY-2 cells. *Plant, Cell & Environment* **33**, 174-187.

Salt, D. E., Prince, R. C., and Pickering, I. J. (2002). Chemical speciation of accumulated metals in plants: evidence from X-ray absorption spectroscopy. *Microchemical Journal* **71**, 255-259.

Salt, D. E., Prince, R. C., Pickering, I. J., and Raskin, I. (1995). Mechanisms of cadmium mobility and accumulation in *Indian mustard*. *Plant Physiology* **109**, 1427-1433.

Samantaray, S., Rout, G. R., and Das, P. (1999). *In vitro* selection and regeneration of zinc tolerant calli from *Setaria italica* L. *Plant Science* **143**, 201-209.

Samantaray, S., Rout, G. R., and Das, P. (2001). Induction, selection and characterization of Cr and Ni-tolerant cell lines of *Echinochloa colona* (L.) Link *in vitro*. *Journal of Plant Physiology* **158**, 1281-1290.

Santandrea, G., Schiff, S., and Bennici, A. (1998). Effects of manganese on *Nicotiana* species cultivated *in vitro* and characterization of regenerated Mn-tolerant tobacco plants. *Plant Science* **132**, 71-82.

Satarug, S., Garrett, S. H., Sens, M. A., and Sens, D. A. (2010). Cadmium, environmental exposure, and health outcomes. *Environmetanl Health Perspective* **118**, 182-190.

Schoeffl, F., and Key, J. L. (1982). An analysis of mRNAs for a group of heat shock proteins of soybean using cloned cDNAs. *Journal of Molecular and Applied Genetics* **1**, 301-314.

Schutzendubel, A., and Polle, A. (2002). Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *Journal of Experimental Botany* **53**, 1351-1365.

Schützendübel, A., and Polle, A. (2002). Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. *Journal of Experimental Botany* **53**, 1351-1365.

Seregin, I. V., and Ivanov, V. B. (2001). Physiological aspects of cadmium and lead toxic effects on higher plants. *Russian Journal of Plant Physiology* **48**, 523-544.

Sharma, P., and Dubey, R. S. (2004). Ascorbate peroxidase from rice seedlings: properties of enzyme isoforms, effects of stresses and protective roles of osmolytes. *Plant Science* **167**, 541-550.

Sharma, R. K., and Agrawal, M. (2005). Biological effects of heavy metals: An overview. *Journal of Environmental Biology* **26**, 301-313.

Shekhawat, G. S., Verma, K., Jana, S., Singh, K., Teotia, P., and Prasad, A. (2010). *In vitro* biochemical evaluation of cadmium tolerance mechanism in callus and seedlings of *Brassica juncea*. *Protoplasma* **239**, 31-38.

Singh, B. R., and McLaughlin, M. J. (1999). Cadmium in soils and plants. *Developments in Plant and Soil Sciences* **85**, 257-267.

Singh, N., Ma, L. Q., Srivastava, M., and Rathinasabapathi, B. (2006). Metabolic adaptations to arsenic-induced oxidative stress in *Pteris vittata* L and *Pteris ensiformis* L. *Plant Science* **170**, 274-282.

Skrebsky, E. C., Tabaldi, L. A., Pereira, L. B., Rauber, R., Maldaner, J., Cargnelutti, D., GonÃ§alves, J. F., Castro, G. Y., Shetinger, M. R. C., and Nicoloso, F. T. (2008). Effect of cadmium on growth, micronutrient concentration, and ´-aminolevulinic acid dehydratase and acid phosphatase activities in plants of *Pfaffia glomerata*. *Brazilian Journal of Plant Physiology* **20**, 285-294.

Smith, G. C., and Brennan, E. G. (1983). Cadmium-Zinc interrelationships in tomato plants. *Phytopathology* **73**, 879-882.

Smykalova, I., Vrbova, M., Tejklova, E., Vetrovcova, M., and Griga, M. (2010). Large scale screening of heavy metal tolerance in flax/linseed (*Linum usitatissimum* L.) tested *in vitro*. *Industrial Crops & Products* **32**, 527-533.

Soler, J. S., and Rovira, J. S. (1996). Cadmium in inorganic fertilizers. *In* "Fertilizers and Environment" (C. Rodriguez-Barrueco, ed.), Vol. 66, pp. 541-545. Springer Netherlands.

Stolt, P., Asp, H., and Hultin, S. (2006). Genetic variation in wheat cadmium accumulation on soils with different cadmium concentrations. *Journal of Agronomy & Crop Science* **192**, 201-208.

Stroiński, A., Chadzinikolau, T., Giżewska, K., and Zielezińska, M. (2010). ABA or cadmium induced phytochelatin synthesis in potato tubers. *Biologia Plantarum* **54**, 117-120.

Subramanian, N. K., White, P. J., Broadley, M. R., and Ramsay, G. (2011). The three-dimensional distribution of minerals in potato tubers. *Annals of Botany* **107**, 681-691.

Sugiyama, M., Ae, N., and Arao, T. (2007). Role of roots in differences in seed cadmium concentration among soybean cultivars—proof by grafting experiment. *Plant and Soil* **295**, 1-11.

Sugiyama, M., Ae, N., and Hajika, M. (2011). Developing of a simple method for screening soybean seedling cadmium accumulation to select soybean genotypes with low seed cadmium. *Plant and Soil* **341**, 413-422.

Sun, J., Cui, J., Luo, C., Gao, L., Chen, Y., and Shen, Z. (2013). Contribution of cell walls, nonprotein thiols, and organic acids to cadmium resistance in two cabbage varieties. *Archives of Environmental Contamination and Toxicology* **64**, 243-252.

Tanhuanpää, P., Kalendar, R., Schulman, A. H., and Kiviharju, E. (2007). A major gene for grain cadmium accumulation in oat *(Avena sativa* L.). *Genome* **50**, 588-594.

Taylor, M., Gibb, R., Willoughby , J., Hewitt, A., and Arnold, G. (2007). "Soil maps of cadmium in New Zealand," Ministry of Agriculture and Forestry. 55.

Toppi, L. S. d., and Gabbrielli, R. (1999). Response to cadmium in higher plants. *Environmental and Experimental Botany* **41**, 105-130.

True, R., Hogan, J., Augustin, J., Johnson, S. J., Teitzel, C., Toma, R. B., and Shaw, R. L. (1978). Mineral composition of freshly harvested potatoes. *American Potato Journal* **55**, 511-519.

Uraguchi, S., Kiyono, M., Sakamoto, T., Watanabe, I., and Kuno, K. (2009). Contributions of apoplasmic cadmium accumulation, antioxidative enzymes and induction of phytochelatins in cadmium tolerance of the cadmium-accumulating cultivar of black oat (*Avena strigosa* Schreb.). *Planta* **230**, 267-276.

Van der Ent, A., Baker, A. M., Reeves, R., Pollard, A. J., and Schat, H. (2013). Hyperaccumulators of metal and metalloid trace elements: Facts and fiction. *Plant Soil* **362**, 319-334.

van Gestel, C. A. M. (2008). Physico-chemical and biological parameters determine metal bioavailability in soils. *Science of The Total Environment* **406**, 385-395.

Van Sint Jan, V., Costa de Macedo, C., Kinet, J.-M., and Bouharmont, J. (1997). Selection of Al-resistant plants from a sensitive rice cultivar, using somaclonal variation, *in vitro* and hydroponic cultures. *Euphytica* **97**, 303-310.

Vangronsveld, J., and Valcke, R. (2007). Subcellular localization of cadmium in roots and leaves of *Arabidopsis thaliana*. *New Phytologist* **173**, 495-508.

Vázquez, S., Goldsbrough, P., and Carpena, R. O. (2006). Assessing the relative contributions of phytochelatins and the cell wall to cadmium resistance in white lupin. *Physiologia Plantarum* **128**, 487-495.

Verbruggen, N., and Hermans, C. (2008). Proline accumulation in plants: a review. *Amino Acids* **35**, 753-759.

Viehweger, K. (2014). How plants cope with heavy metals. *Botanical Studies* **55**, 35-35.

Visarada, K. B. R. S., Sailaja, M., and Sarma, N. P. (2002). Effect of callus induction media on morphology of embryogenic calli in rice genotypes. *Biologia Plantarum* **45**, 495-502.

Vögeli-Lange, R., and Wagner, G. J. (1990). Subcellular localization of cadmium and cadmium-binding peptides in tobacco leaves: Implication

of a transport function for cadmium-binding peptides. *Plant Physiology* **92**, 1086-1093.

Vollenweider, P., Cosio, C., Günthardt-Goerg, M. S., and Keller, C. (2006). Localization and effects of cadmium in leaves of a cadmium-tolerant willow (*Salix viminalis* L.). *Environmental and experimental botany* **58**, 25-40.

Von Arnold, S., Sabala, I., Bozhkov, P., Dyachok, J., and Filonova, L. (2002). Developmental pathways of somatic embryogenesis. *Plant Cell, Tissue and Organ Culture* **69**, 233-249.

Vyver, C. v. d., Conradie, T., Kossmann, J., and Lloyd, J. (2013). *In vitro* selection of transgenic sugarcane callus utilizing a plant gene encoding a mutant form of acetolactate synthase. *In Vitro Cellular & Developmental Biology - Plant* **49**, 198-206.

Wang, Q.-M., and Wang, L. (2012). An evolutionary view of plant tissue culture: somaclonal variation and selection. *Plant Cell Reports* **31**, 1535-1547.

Wang, X., Zhang, Z.-W., Tu, S.-H., Feng, W.-Q., Xu, F., Zhu, F., Zhang, D.-W., Du, J.-B., Yuan, S., and Lin, H.-H. (2013). Comparative study of four rice cultivars with different levels of cadmium tolerance. *Biologia* **68**, 74-81.

Wang, Y., Jiang, X., Li, K., Wu, M., Zhang, R., Zhang, L., and Chen, G. (2014a). Photosynthetic responses of *Oryza sativa* L. seedlings to cadmium stress: physiological, biochemical and ultrastructural analyses. *BioMetals* **27**, 389-401.

Wang, Y., Xiao, X., Zhang, T., Kang, H., Zeng, J., Fan, X., Sha, L., Zhang, H., Yu, K., and Zhou, Y. (2014b). Cadmium treatment alters the expression of five genes at the Cda1 locus in two soybean cultivars [*Glycine max* (L.) Merr]. *The Scientific World Journal* **2014**, 1-8.

Wanger, G. (1993). Accumulation of cadmium in crop plants and its consequences to human health. *Advances in Agronomy*, 173-212.

Weale, A. (2010). Ethical arguments relevant to the use of GM crops. *New Biotechnology* **27**, 582-587.

Wei, L., Luo, C., Li, X., and Shen, Z. (2008). Copper accumulation and tolerance in *Chrysanthemum coronarium* L. and *Sorghum sudanense* L. *Archives of Environmental Contamination and Toxicology* **55**, 238-246.

Welch, R. M., and Graham, R. D. (2004). Breeding for micronutrients in staple food crops from a human nutrition perspective. *Journal of experimental botany* **55**, 353-364.

Wilson, C. R., Luckman, G. A., Tegg, R. S., Yuan, Z. Q., Wilson, A. J., Eyles, A., and Conner, A. J. (2009). Enhanced resistance to common scab of potato through somatic cell selection in cv. Iwa with the phytotoxin thaxtomin A. *Plant Pathology* **58**, 137-144.

Wilson, C. R., Tegg, R. S., Wilson, A. J., Luckman, G. A., Eyles, A., Yuan, Z. Q., Hingston, L. H., and Conner, A. J. (2010). Stable and extreme resistance to common scab of potato obtained through somatic cell selection. *Phytopathology* **100**, 460-467.

Winterbourn, C. C. (1995). Toxicity of iron and hydrogen peroxide: the Fenton reaction. *Toxicology Letters* **82–83**, 969-974.

World Health Organisation (1992). "Cadmium." Environmental health criteria 134. Geneva, Switzerland.

Wu, C. H., Wood, T. K., Mulchandani, A., and Chen, W. (2006). Engineering plant-microbe symbiosis for rhizoremediation of heavy metals. *Applied and Environmental Microbiology* **72**, 1129-1134.

Wu, F., and Zhang, G. (2002). Genotypic variation in kernel heavy metal concentrations in bareley and as affected by soil factors. *Journal of Plant Nutrition* **25**, 1163-1173.

Wuana, R. A., and Okieimen, F. E. (2011). Heavy metals in contaminated soils: a review of sources, chemistry, risks and best available strategies for remediation. *Ecology* **2011**, 1-20.

Xu, J., Yin, H., and Li, X. (2009). Protective effects of proline against cadmium toxicity in micropropagated hyperaccumulator, *Solanum nigrum* L. *Plant Cell Reports* **28**, 325-333.

Xu, X.-y., Shi, G.-x., Wang, J., Zhang, L.-l., and Kang, Y.-n. (2011). Copper-induced oxidative stress in *Alternanthera philoxeroides* callus. *Plant Cell, Tissue and Organ Culture (PCTOC)* **106**, 243-251.

Yoon, K. S., and Leung, D. W. M. (2004). Relative importance of maltose and sucrose supplied during a 2-step potato microtuberization process. *Acta Physiol. Plant.* **26**, 47-52.

Yu, H., Wang, J., Fang, W., Yuan, J., and Yang, Z. (2006). Cadmium accumulation in different rice cultivars and screening for pollution-safe cultivars of rice. *Science of The Total Environment* **370**, 302-309.

Zeng, F., Mao, Y., Cheng, W., Wu, F., and Zhang, G. (2008). Genotypic and environmental variation in chromium, cadmium and lead concentrations in rice. *Environmental Pollution* **153**, 309-314.

Zhan, J., Wei, S., Niu, R., Li, Y., Wang, S., and Zhu, J. (2013). Identification of rice cultivar with exclusive characteristic to Cd using a field-polluted soil and its foreground application. *Environmental Science and Pollution Research* **20**, 2645-2650.

Zhang, G., Fukami, M., and Sekimoto, H. (2002). Influence of cadmium on mineral concentrations and yield components in wheat genotypes differing in Cd tolerance at seedling stage. *Field Crops Research* **77**, 93-98.

Zhang, H., Zhang, X., Li, T., and Huang, F. (2014). Variation of cadmium uptake, translocation among rice lines and detecting for potential cadmium-safe cultivars. *Environmental Earth Sciences* **71**, 277-286.

Zhang, K., Yuan, J., Kong, W., and Yang, Z. (2013). Genotype variations in cadmium and lead accumulations of leafy lettuce (*Lactuca sativa* L.) and screening for pollution-safe cultivars for food safety. *Environmental Science: Processes & Impacts* **15**, 1245-1255.

Zhang, Y., and Donnelly, D. (1997). *In vitro* bioassays for salinity tolerance screening of potato. *Potato Research* **40**, 285-295.

Zhao, M.-X., Sun, H.-Y., Ji, R.-R., Hu, X.-H., Sui, J.-M., Qiao, L.-X., Chen, J., and Wang, J.-S. (2013). *In vitro* mutagenesis and directed screening for salt-tolerant mutants in peanut. *Euphytica* **193**, 89-99.

Zheng, R.-L., Li, H.-F., Jiang, R.-F., and Zhang, F.-S. (2008). Cadmium accumulation in the edible parts of different cultivars of radish, *Raphanus sativus* L., and carrot, *Daucus carota* var. sativa, grown in a Cd-contaminated soil. *Bulletin of Environmental Contamination and Toxicology* **81**, 75-79.

Zhou, J., and Goldsbrough, P. B. (1994). Functional homologs of fungal metallothionein genes from *Arabidopsis*. *Plant Cell* **6**, 875-884.

Zhou, Y., Xue, M., Yang, Z., Gong, Y., Yuan, J., Zhou, C., and Huang, B. (2013). High cadmium pollution risk on vegetable amaranth and a

selection for pollution-safe cultivars to lower the risk. *Frontiers of Environmental Science & Engineering* **7**, 219-230.

Zhu, E., Liu, D., Li, J. G., Li, T. Q., Yang, X. E., He, Z. L., and Stoffella, P. J. (2010). Effect of nitrogen fertilizer on growth and cadmium accumulation in *Sedum alfredii* hance. *Journal of Plant Nutrition* **34**, 115-126.

Zhu, Y., Yu, H., Wang, J., Fang, W., Yuan, J., and Yang, Z. (2007). Heavy metal accumulations of 24 asparagus bean cultivars grown in soil contaminated with Cd alone and with multiple metals (Cd, Pb, and Zn). *Journal of Agricultural and Food Chemistry* **55**, 1045-1052.

Zorrig, W., El Khouni, A., Ghnaya, T., Davidian, J. C., Abdelly, C., and Berthomieu, P. (2013). Lettuce (*Lactuca sativa*): a species with a high capacity for cadmium (Cd) accumulation and growth stimulation in the presence of low Cd concentrations. *Journal of Horticultureal Science & Biotechnology* **88**, 783-789.

Appendix

MURASHIGE & SKOOG STOCK SOLUTIONS

1) Major Salts (10x)

		1 litre	2 litres
NH_4NO_3		16.5 g	33 g
KNO_3		19.0 g	38 g
$CaC1_2.2H_2O$		4.4 g	8.8 g
$MgSO_4.7H_2O$		3.7 g	7.4 g
KH_2PO_4		1.7 g	3.4 g
dH_2O - bring vol. to	→	1 litre	2 litres

Label and store at 4° C

2) Minor salts (100x)

		1 litre	2 litres
KI		0.083 g	0.166 g
H_3BO_3		0.620 g	1.240 g
$MnSO_4.4H_2O$		2.230 g	4.460 g
$ZnSO_4.7H_20$		0.860 g	1.720 g
$CuSO_45H_2O$		0.0025 g	0.005 g
$CoCl_2.6H_2O$		0.0025 g	0.005 g
$Na_2MoO_4.2H_2O$		0.025 g	0.050 g
dH_2O - bring vol. to	→	1 litre	2 litres

Label & store at 4°C

3) Organic supplement (100x)

		500 ml	1 litre
myo-inositol		5,000 mg	10,000 mg
Nicotinic acid		25 mg	50 mg
pyridoxine-HC1		25 mg	50 mg
thiamine-HC1		5 mg	10 mg
glycine		100 mg	200 mg
dH_2O - bring vol. to	→	500 ml	1 litre

Label and store at 4°C

Iron Stock

Soln A:	$FeSO_4.7H_2O$	1.39 g in 200 ml dH_2O
Soln B:	$Na_2EDTA.2H_2O$	1.865 g in 200 ml dH_2O

Mix soln A and soln B
Adjust volume to 500 ml
Store in dark bottle at 4°C